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Impact of ozone exposure on the phagocytic activity of human surfactant protein A (SP-A) and SP-A variants

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Abstract

Surfactant protein A (SP-A) enhances phagocytosis of *Pseudomonas aeruginosa*. SP-A1 and SP-A2 encode human (h) SP-A; SP-A2 products enhance phagocytosis more than SP-A1. Oxidation can affect SP-A function. We hypothesized that in vivo and in vitro ozone-induced oxidation of SP-A (as assessed by its carbonylation level) negatively affects its function in phagocytosis (as assessed by bacteria cell association). To test this, we used *P. aeruginosa*, rat alveolar macrophages (AMs), hSP-As with varying levels of in vivo (natural) oxidation, and ozone-exposed SP-A2 (1A, 1A⁰) and SP-A1 (6A², 6A⁴) variants. SP-A oxidation levels (carbonylation) were measured; AMs were incubated with bacteria in the presence of SP-A, and the phagocytic index was calculated. We found: 1) the phagocytic activity of hSP-A is reduced with increasing levels of in vivo SP-A carbonylation; 2) in vitro ozone exposure of hSP-A decreases its function in a dose-dependent manner as well as its ability to enhance phagocytosis of either gram-negative or gram-positive bacteria; 3) the activity of both SP-A1 and SP-A2 decreases in response to in vitro ozone exposure of proteins with SP-A2 being affected more than SP-A1. We conclude that both in vivo and in vitro oxidative modifications of SP-A by carbonylation reduce its ability to enhance phagocytosis of bacteria and that the activity of SP-A2 is affected more by in vitro ozone-induced oxidation. We speculate that functional differences between SP-A1 and SP-A2 exist in vivo and that the redox status of the lung microenvironment differentially affects function of SP-A1 and SP-A2.

Keywords

air pollution; host defense; carbonylation; macrophage

The lung effectively provides initial protection against a large number of harmful inhaled pathogens, particles, allergens, air pollutants, and other irritants. Pulmonary surfactant, which lines the entire surface of the lung, plays a key role in its immune defense. Maintenance of the integrity of the respiratory tract and the surfactant system is an important mechanism for the regulation of host defense.

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Surfactant protein A (SP-A) is a major surfactant host defense component and belongs to a group of mammalian lectins, the collectins, that are involved in innate immunity (13,27). SP-A has been shown to be involved in the stimulation of chemotaxis of macrophages (62), the enhancement of phagocytosis of bacteria (10,25,36,38,39), the proliferation of immune cells (2,31), the linkage of innate and adaptive immunity (4), and in the modulation of the production of proinflammatory cytokines (3,30,60,61). Genetically modified mice lacking SP-A are more susceptible to challenge with experimental pneumonia than wild-type mice (34).

The human (h) SP-A locus consists of two functional genes, SP-A1 and SP-A2, and a pseudogene. A number of variants for each SP-A gene have been characterized based on their coding sequence differences (9,23). The frequency of the various SP-A1 and SP-A2 variants differs in the general human population (9). The mature SP-A molecule consists of four domains: NH₂-terminal, collagen-like, neck, and carbohydrate recognition domain (CRD). Amino acid differences that distinguish between SP-A1 and SP-A2 variants are localized in the collagen-like domain. Nucleotide differences that do or do not change the encoded amino acid specific to a given variant are located within the coding regions for the signal peptide, collagen-like, and CRD domains (14).

SP-A variants have been identified with both qualitative (functional, biochemical, and/or structural) and quantitative (regulatory) differences. Qualitative differences include differences in their ability to stimulate phagocytosis (38,39), bind carbohydrates (43), inhibit surfactant secretion (56), and stimulate production of TNF- α by macrophage-like THP-1 cells (20,60,61), as well as differences in their aggregation and oligomerization properties (15,50, 56). However, no differences have been observed between SP-A1 and SP-A2 in their ability to inhibit hemagglutination activity of influenza A virus (40). Quantitative differences include differences between the SP-A1 and SP-A2 genes and/or variants in basal mRNA levels and in response to dexamethasone (22,33,48,58) and in protein levels in bronchoalveolar lavage (BAL) fluids from different individuals (52). Mechanisms involving NF- κ B activation (21, 28) and mRNA stability and translational control (57) may contribute to quantitative differences.

Air pollutants influence the structure and function of the surfactant system (42). Ozone, a major component of air pollution, is a strong oxidizing agent and can affect surfactant function. SP-A, an important component of the surfactant system, showed impaired functional abilities after exposure to oxidants, including ozone. This is likely to be the result of oxidative modification of SP-A, which may include irreversible oxidation of various amino acids by carbonylation as well as methionine oxidation, tyrosine nitration, a variety of reversible and irreversible oxidative modifications of cysteine sulf-hydryls, and others. Carbonyl content is often used as an index of oxidation (24,35) although it only measures some of the modifications. Ozone-induced oxidation of SP-A reduces its ability to interact with alveolar macrophages (AMs; Ref. 46), inhibits phosphatidylcholine secretion from alveolar type II cells (45,56), and has an impact on cytokine production in THP-1 cells (61) via ineffective activation of the NF- κ B cell signaling pathway (21). After ozone exposure of SP-A, its aggregation pattern, absorption spectra, gel electrophoretic pattern (50,56), and SP-A-dependent extracellular surfactant morphology (45,46) are also changed. Thus impairment of SP-A activity via oxidation may be one of the mechanisms that contributes to the increased risk of pneumonia when ozone levels are elevated (11,37,47).

Variability in the susceptibility to ozone exposure has been observed among humans (8,18, 32). It is possible that the increased sensitivity to infection that some individuals exhibit after ozone exposure, as assessed by higher risk of common infections in areas with highly polluted air (42), may reflect the involvement of intrinsic or genetic factors and/or the functional impairment of oxidant-exposed SP-A. Different individuals may possess different

combinations of SP-A variants and proportions of SP-A1 and SP-A2 content (52). Because different SP-A variants have been identified with qualitative and quantitative differences, and functional differences between SP-A1 and SP-A2 have been observed (15,20,39,43,56,60, 61), we speculate that the ratio of SP-A1 to SP-A2 is a better indicator of the overall ability of hSP-A to enhance phagocytosis in the lung than the total SP-A content. This, in turn, may better help to explain individual differences in susceptibility to pulmonary disease.

In the present study, we investigated the hypothesis that the function of certain SP-A1 and/or SP-A2 variants to enhance phagocytosis (as assessed by bacteria cell association) is differentially compromised in response to ozone-induced oxidation. Thus we studied for the first time the effect of oxidation on the ability 1) of hSP-A to enhance phagocytosis of gram-positive and gram-negative bacteria and 2) of SP-A1 and SP-A2 variants to enhance phagocytosis of *Pseudomonas aeruginosa*. The impact of both in vivo (in the lung) and in vitro (by ozone exposure) oxidation on SP-A function was studied. hSP-As from BAL and in vitro-expressed SP-A variants using mammalian and insect cell expression systems were used. The latter helped discern the importance of SP-A post-translational modifications on its function in response to ozone-induced oxidation.

METHODS

Reagents and Media

Tryptic soy agar and RPMI 1640 (HEPES modification) were from Sigma (St. Louis, MO), and Dulbecco's PBS was from Invitrogen Life Technologies (Grand Island, NY). Sterile saline solution (0.9% NaCl) was purchased from Baxter Healthcare (Deerfield, IL). BAL fluid was obtained from patients with alveolar proteinosis (AP) who were undergoing routine therapeutic lavage. All procedures involving animals were carried out according to protocol approved by The Pennsylvania State University Institutional Animal Care and Use Committee. The protocol for human specimen collection was approved by the Institutional Review Board.

Growing and Preparation of Bacteria

A nonmucoid *P. aeruginosa* strain (ATCC 39018) or *Staphylococcus aureus* strain (ATCC 25923) was obtained from the American Type Culture Collection (Rockville, MD). Bacteria suspensions were prepared as described previously (38). Briefly, bacteria were grown overnight (20–24 h) on tryptic soy agar plates at 30°C and resuspended in the RPMI medium to a required bacterial concentration. The colony-forming units (CFU) per milliliter value was determined with a calibration curve based on the optical density (OD) of a bacterial suspension at 660 nm. The bacterial suspension was then used immediately, and an aliquot was spread on agar plates to control for the quantification of bacteria.

Purification of SP-A from Human BAL

SP-A was purified (16) from the BAL fluid obtained from a patient with AP (AP BAL hSP-A) as described previously (19). The concentration of SP-A was determined using the Micro BCA Protein Assay (Pierce, Rockford, IL) using RNase A as a standard, and the purity of the SP-A preparation was verified by 2-D-PAGE followed by silver staining.

Preparation and Purification of hSP-A Genetic Variants Expressed in the Baculovirus-Mediated Insect Cell System

hSP-A genetic variants (for SP-A1, 6A⁴; for SP-A2, 1A) were expressed using the baculovirus-mediated insect system as described previously (60). Briefly, SP-A variant expression was achieved with inoculation of a virus containing the SP-A variant gene into insect cells growing in Sf-900 II SFM (Invitrogen Life Technologies). The inoculated cells were cultured at 27°C

with shaking at a starting density of 2×10^6 cells/ml. Supernatants were harvested 72 h after inoculation, and proteins were purified by mannose-affinity chromatography as described previously (61). The purified SP-A variants were then dialyzed against 5 mM Tris (pH 7.5) with three changes of dialysis buffer and examined by silver staining of SDS-PAGE gels and by Western blotting. The protein concentration was determined using the Micro BCA Protein Assay and RNase A as a standard.

Preparation and Purification of hSP-A Genetic Variants Expressed in the Mammalian (CHO) Cell System

hSP-A variants (for SP-A1, 6A² and 6A⁴; for SP-A2, 1A⁰) were expressed from transfected mammalian Chinese hamster ovary (CHO)-K1 cells as described previously (61). Briefly, both human SP-A1 and SP-A2 cDNAs were cloned into the expression vector pEE14. SP-A gene transcription was driven by a cytomegalovirus promoter. The recombinant constructs with SP-A variants were transfected into CHO cells. Stably transfected cell lines were established based on SP-A expression. SP-A variants were purified from the media of cultivated CHO cells using mannose-affinity chromatography as described previously (61). Mammalian cell-expressed SP-A variants were dialyzed and checked for purity as described above for insect cell-expressed variants.

In Vitro Exposure of SP-A to Ozone and Detection of the Level of Protein Carbonylation

SP-A preparations were exposed to ozone in 24-well tissue culture plates (100 μ l/well) as described previously (53). The plates were exposed to ozone at 0.01, 0.1, 1, and 10 ppm or to filtered air (FA) for 4 h. An ozone concentration of 1 ppm for 4 h of exposure was found before to be optimal for SP-A oxidation (53) as measured by carbonylation. Protein carbonylation levels were measured with the OxyBlot Oxidized Protein Detection Kit (Chemicon, Temecula, CA), which detects carbonyl groups introduced into protein by oxidative reactions. Briefly, carbonyl groups in the ozone-exposed proteins were derivatized with 2,4-dinitrophenyl hydrazine (DNPH), 200 ng of DNPH-derivatized protein were blotted to nitrocellulose, and immunodetection of DNPH-derivatized proteins was performed with anti-DNPH rabbit primary IgG and then with goat anti-rabbit secondary IgG conjugated with horseradish peroxidase. Blots were exposed to XAR films following enhanced chemiluminescent detection and quantified by densitometry. Blots were exposed for different time intervals to obtain optimal exposures. Carbonylation levels are expressed as the OD for each spot multiplied by its area.

Isolation of Rat AMs

Male pathogen-free Sprague-Dawley rats (250–300 g; Harlan, Indianapolis, IN) were used as a source for AMs. AMs were isolated as described previously (38). Briefly, animals were anesthetized with an intramuscular injection of a mixture of Ketamine HCl (Ketaset, Fort Dodge Animal Health) and xylazine (Xyla-Ject; Phoenix Pharmaceuticals, St. Joseph, MO). Rats were killed and exsanguinated, the tracheas were cannulated, and the lungs lavaged three times with sterile saline (0.9% NaCl). Lavages were centrifuged, and the cell pellets were washed three times with RPMI medium. Cells were counted using a hemocytometer and resuspended in RPMI medium at 1×10^6 cells/ml. The viability of the cells was determined by Trypan blue dye exclusion, and only cell suspensions with >95% viable cells were used.

Phagocytic Index Calculation

To calculate the phagocytic index (PI), we assessed phagocytosis (attachment and internalization steps of phagocytosis or cell association) of *P. aeruginosa* by AMs. The assay was performed as described previously by others (5,7,44) and us (38,39). Briefly, suspensions of rat AMs (10^6 cells/ml) and bacteria (10^7 CFU/ml) in RPMI medium were mixed (50 + 50

μl) to obtain a 1:10 ratio of cells to bacteria. SP-A was then immediately added to the mixture, and the mixture was incubated on a LABQUAKE shaker for 1 h at 37°C for the phagocytosis assay. One milliliter of ice-cold PBS was then added to stop phagocytosis. AMs were sedimented by centrifugation at 4°C at 250 g for 10 min and washed twice more with 1 ml of cold PBS followed by sedimentation. Final suspensions of AMs were resuspended in 200 μl of PBS and applied to slides by cyto centrifugation. The slides were stained using the Hema-3 Stain Kit (Fisher Scientific, Pittsburgh, PA) for analysis by light microscopy. Two hundred randomly selected AMs per each experimental point were analyzed at ×1,000 magnification under oil immersion (7). The PI was calculated according to the following formula: the percent of bacteria-positive macrophages (cells that phagocytized at least 1 bacterium) multiplied by the average number of bacteria per bacteria-positive macrophage (5). The values for the PI were expressed as percent of the negative control (i.e., without SP-A), which was set to 100%.

Statistics

Paired *t*-tests were performed for comparison of the effects of SP-As used at the same concentration, and two-sample *t*-tests were used for comparison of the effects of SP-As at different concentrations. Data were expressed as means ± SE for three or more independent experiments. Differences were considered significant when $P < 0.05$.

RESULTS

To evaluate the effect of SP-A on the phagocytosis of live *P. aeruginosa* by rat AMs, we used a traditional light microscopy method (38,39) that allows assessment of two steps of the phagocytosis process (attachment and internalization or cell association) together. As we (39) have shown before, cell association (PI) determined by light microscopy correlates with internalization of bacteria as assessed by FACS analysis, and thus both characterize phagocytosis.

Impact of SP-A Oxidation on its Ability to Enhance Phagocytosis of *P. aeruginosa* by Rat AMs

In vivo (in the lung) SP-A oxidation—To test the effect of in vivo (natural) oxidation of hSP-A on its phagocytic activity, we tested 10 different SP-A preparations with different degrees of carbonylation, isolated from 6 individuals (Fig. 1). From *individuals* 2 and 6, we studied 3 different protein preparations from each (2-1, 2-2, and 2-3 and 6-1, 6-2, 6-3, respectively). No significant differences were found among the activities of the different SP-A preparations isolated from either *individual* 2 or 6. When the results for *preparations* 2-1, 2-2, and 2-3 (3 preparations from the same individual) were pooled together and averaged, and the results for *preparations* 6-1, 6-2, and 6-3 (3 preparations from the same individual) were also pooled together and averaged, we found that the activity of SP-A 6 significantly ($P < 0.05$) differed from the activities of *preparation* 1, 2, 3, 4, or 5, with no significant differences observed among activities of SP-A preparations from *individuals* 1, 2, 3, 4, and 5. Moreover, when each protein preparation of *samples* 2 and 6 was treated as a separate sample, significant differences ($P < 0.05$) were found between the activities of SP-A 6-1 and SP-As 1 and 2-1, between SP-A 6-2 and SP-As 1 and 2-1, and between SP-A 6-3 and SP-As 1, 2-1, 2-2, 2-3, 3, 4, and 5. Next, a correlation of the in vivo oxidation (carbonylation) levels of SP-A and its phagocytic activity was determined using the Pearson product-moment correlation coefficient. We found a negative correlation (correlation coefficient -0.7 for all separate samples and -0.9 for pooled triplicate samples from *individuals* 2 and 6 shown in Fig. 1, *A* and *B*, respectively) at a significance level of $P < 0.05$. A decrease in the enhancement of phagocytosis was observed with increasing carbonylation of the SP-A. It should be noted that the carbonylation level (OD × area) is not an absolute value (but rather a relative value) and can vary among experiments depending on the exposure period for the enhanced chemiluminescent blot.

In vitro exposure of SP-A to ozone—To study the dependence of SP-A-enhanced phagocytosis on the levels of SP-A carbonylation, we exposed SP-A in vitro to increasing concentrations of ozone. SP-A 6-2 (Fig. 2) had a low natural carbonylation level and was chosen for subsequent experimentation. This SP-A was exposed to different concentrations of ozone: 0.01, 0.1, 1, and 10 ppm. Also, as a control, FA was used to treat SP-A in vitro. Ozone exposure time was 4 h.

We found that the PI of untreated SP-A differed significantly from the PIs of all those exposed to ozone. FA-exposed SP-A (used as an additional control) was more active than SP-A exposed to the 0.1-, 1-, and 10-ppm concentrations of ozone, and SP-A exposed to 0.01 ppm of ozone was more active than SP-A exposed to 1 ppm of ozone. We observed that with increased ozone-induced carbonylation, the PI was progressively decreased (PIs were 820 ± 16 and 744 ± 46 for untreated and FA-exposed, respectively, and PIs were 672 ± 27 , 619 ± 40 , 405 ± 12 , and 389 ± 68 for SP-A exposed to ozone at 0.01, 0.1, 1 and 10 ppm, respectively; Fig. 2). A strong negative correlation (Pearson product-moment correlation coefficient: -0.9) was found, at a significance level of $P < 0.05$, between the level of ozone-induced carbonylation of SP-A and its ability to enhance phagocytosis.

Phagocytosis of *S. aureus* by Rat AMs in the Presence of Ozone-Exposed hSP-A

We tested the ability of in vitro ozone-exposed hSP-A to enhance phagocytosis of different bacteria: gram-positive (*S. aureus*) and gram-negative (*P. aeruginosa*). For both types of bacteria, the same effect was found: ozone-exposed SP-A was significantly less active than untreated SP-A (Fig. 3).

Effect of In Vitro Ozone Exposure of CHO Cell-Expressed SP-A1 and SP-A2 Variants on their Ability to Stimulate Phagocytosis of *P. aeruginosa* by Rat AMs

To assess the effect of in vitro ozone exposure on the ability of SP-A1 and SP-A2 to enhance phagocytosis of *P. aeruginosa*, we used SP-A variants expressed in the mammalian (CHO) expression system where posttranslational modifications are more like these found in native hSP-A. We used untreated, FA-exposed, and ozone-exposed SP-A1 ($6A^4$) and SP-A2 ($1A^0$) variants at three different concentrations: 5, 10, and 20 $\mu\text{g/ml}$ (Fig. 4). As a positive control, hSP-A was used at a single 5 $\mu\text{g/ml}$ concentration. No significant differences were observed between untreated and FA-exposed SP-As, which were both used as controls. This indicates that airflow considerations are not a factor and thus not relevant in the present study. Therefore, in some further analyses, only untreated SP-A was used. We found that all SP-A preparations were significantly ($P < 0.05$) more active than the negative control (without SP-A) with the exception of ozone-exposed $1A^0$ at 5 $\mu\text{g/ml}$ and $6A^4$ at 5 and 10 $\mu\text{g/ml}$ concentrations. In general, all preparations were more active at 20 $\mu\text{g/ml}$ compared with the lower SP-A (5 $\mu\text{g/ml}$) concentration used.

Results for the $1A^0$ Variant

For the $1A^0$ variant, the PI values for untreated, FA-exposed, and ozone-exposed SP-A were, respectively, as follows: $214\% \pm 13\%$, $171\% \pm 14\%$, and $95\% \pm 6\%$ at the 5 $\mu\text{g/ml}$ concentration; $213\% \pm 19\%$, $194\% \pm 5\%$, and $125\% \pm 6\%$ at the 10 $\mu\text{g/ml}$ concentration; and $286\% \pm 8\%$, $266\% \pm 15\%$, and $181\% \pm 11\%$ at the 20 $\mu\text{g/ml}$ concentration. All untreated and FA- and ozone-exposed $1A^0$ were significantly more active at a 20 $\mu\text{g/ml}$ concentration compared with 5 $\mu\text{g/ml}$. Both untreated and FA-exposed $1A^0$ SP-As were more active at 20 $\mu\text{g/ml}$ than at 10 $\mu\text{g/ml}$. Ozone-exposed $1A^0$ was significantly less active than either untreated or FA-exposed SP-A at all concentrations tested.

Results for the 6A⁴ Variant

For the 6A⁴ variant, the PI values for untreated, FA-exposed, and ozone-exposed SP-A were, respectively, as follows: 119% $\pm$ 4%, 120% $\pm$ 5%, and 102% $\pm$ 15% for the 5 μ g/ml concentration; 133% $\pm$ 5%, 128% $\pm$ 3%, and 108% $\pm$ 12% for the 10 μ g/ml concentration; 173% $\pm$ 10%, 157% $\pm$ 8%, and 124% $\pm$ 5% for the 20 μ g/ml concentration. Both untreated and FA-exposed 6A⁴ were significantly more active at the 20 μ g/ml concentration compared with the 5 μ g/ml concentration. The activities of untreated, FA-exposed, and ozone-exposed 6A⁴ at 10 μ g/ml also did not differ among themselves nor did they differ from those at 10 μ g/ml. The activities of untreated, FA-exposed, and ozone-exposed 6A⁴ at 10 μ g/ml also did not differ among themselves. The untreated 6A⁴ was found to be significantly more active at 20 μ g/ml than at 10 μ g/ml. Ozone-exposed 6A⁴ was less active than either untreated or FA-exposed SP-A only at the 20 μ g/ml concentration.

Results for the hSP-A

For hSP-A, the PI values for untreated, FA-exposed, and ozone-exposed SP-A were 820% $\pm$ 19%, 754% $\pm$ 54%, and 389% $\pm$ 61%, respectively. Ozone-exposed hSP-A was significantly less active than either untreated or FA-exposed SP-A.

Comparison of SP-A1 and SP-A2 Activities

Comparison of SP-A1 and SP-A2 activities at the same concentration indicated that the PIs between untreated and FA-exposed 1A⁰ and 6A⁴ differed at all the SP-A concentrations tested. Ozone-exposed 6A⁴ differed from ozone-exposed 1A⁰ at the 20 μ g/ml concentration (Fig. 4). No significant differences were found between SP-A1 and SP-A2 activities after ozone exposure at SP-A concentrations of 5 and 10 μ g/ml (108% and 86%, respectively), whereas differences were observed when untreated or FA-exposed variants were used (Table 1). When 1A⁰ activity (used at its minimum concentration, 5 μ g/ml) was compared with 6A⁴ activity at the maximum concentration (20 μ g/ml), no differences were found for untreated or FA-exposed proteins. However, at the same concentrations (5 μ g/ml for 1A⁰ and 20 μ g/ml for 6A⁴), ozone-exposed proteins significantly differed in their activities; the 6A⁴ exhibited higher activity ($P < 0.05$) than 1A⁰ (Fig. 4).

Comparison of the ratios of the PI values of ozone-exposed-to-untreated or ozone-exposed-to-FA-exposed showed that the 1A⁰ activity was significantly affected by ozone at all concentrations tested, whereas the 6A⁴ activity was affected by ozone exposure only at the higher (20 μ g/ml) concentration (Table 2). Because the activity of untreated and FA-exposed 1A⁰ is considerably higher than the corresponding activity of 6A⁴, we compared ratios for 1A⁰ at the minimum (5 μ g/ml) concentration with the ratios for 6A⁴ activity at all concentrations tested (5, 10, and 20 μ g/ml). We observed: 1) the value of 1A⁰ ozone-exposed-to-untreated was significantly lower than all comparable values for 6A⁴ (at 5, 10, and 20 μ g/ml concentrations); and 2) the value of ozone-exposed-to-FA-exposed was significantly lower for 1A⁰ than that for 6A⁴ at 20 μ g/ml concentration (Table 2). We observed the same trend of the effect of ozone exposure on the ability of another SP-A1 variant (6A²) to stimulate the phagocytosis and found (as for 6A⁴ variant) that the ozone/FA PI ratio for 6A² was also higher than that for 1A⁰ (SP-A2) (data not shown). Thus ozone exposure affects SP-A2 (1A⁰) activity more than that of SP-A1 (6A², 6A⁴).

Effect of In Vitro Ozone Exposure of Insect Cell-Expressed SP-A1 and SP-A2 Variants on their Ability to Stimulate Phagocytosis of *P. aeruginosa* by Rat AMs

Following phagocytosis assays, the PI values were compared between ozone-exposed SP-As and their untreated SP-A preparations. Because insect cell-expressed SP-A variants have been shown to be considerably less active than hSP-A (38,39), 100 μ g/ml for insect cell-derived SP-

A variants and 5 $\mu\text{g/ml}$ for hSP-A were used. The carbonylation levels of SP-As and PIs are shown in Fig. 5. The PIs calculated for untreated and ozone-exposed SP-A proteins were, respectively: for hSP-A, $786\% \pm 44\%$ and $519\% \pm 32\%$; for 1A, $266\% \pm 15\%$ and $166\% \pm 11\%$; for 6A⁴, $126\% \pm 5\%$ and $165\% \pm 18\%$. A significant reduction in the ability of SP-As to enhance phagocytosis after exposure to ozone was observed (at $P < 0.05$) for hSP-A (66%) and 1A (62%) (Table 3). The activity of ozone-exposed 6A⁴ did not differ significantly from untreated 6A⁴. Although significant differences were seen between the PIs of unexposed 1A and 6A⁴ (PI: 1A > 6A⁴; PI: $266\% > 126\%$), no significant differences were observed between ozone-exposed 1A (PI: $166\% \pm 11\%$) and 6A⁴ (PI: $165\% \pm 18\%$) variant activities. We observed that the value of ozone-exposed-to-untreated ratio for 1A (SP-A2; $63.0\% \pm 5.0\%$) was significantly lower than the comparable value for 6A⁴ (SP-A1; $130.3\% \pm 13.5\%$) (Table 3). This is similar to that noted for CHO cell-expressed SP-A1 and SP-A2 variants (Table 2). Thus ozone exposure affects SP-A2 activity more than that of SP-A1.

Together, we tested three SP-A variants for SP-A1 (CHO cell-expressed 6A⁴ and 6A² and insect cell-expressed 6A⁴) and two SP-A variants for SP-A2 (CHO cell-expressed 1A⁰ and insect cell-expressed 1A) for the effect of ozone exposure on the ability of SP-A1 and SP-A2 variants to stimulate phagocytosis. Figures 4 and 5 and Tables 1, 2, and 3 demonstrate the same effect: ozone has a higher negative impact on SP-A2 activity than on SP-A1 activity at least for the gene-specific variants tested.

DISCUSSION

Ozone, one of the major pollutants in the air, affects lung function and surfactant components including SP-A, an important host defense molecule of the lung. As we (38,39) reported before, the SP-A2 gene products are more effective in their ability to enhance phagocytosis of *P. aeruginosa* by AMs than SP-A1. In this study, we investigated the hypothesis that the ability of SP-A to stimulate phagocytosis of *P. aeruginosa* is affected by in vivo oxidation and in vitro ozone-induced oxidation, as measured by carbonylation, and that the phagocytic activities of SP-A1 and SP-A2 are differentially affected by ozone exposure (carbonylation). For this, we studied the phagocytic activity of 1) SP-As with different levels of in vivo oxidation (carbonylation) that were isolated from BAL of different individuals, 2) SP-A from BAL that was subsequently exposed to ozone, and 3) in vitro expressed SP-A1 and SP-A2 variants after exposure to ozone. We found: 1) there is a negative correlation between the ability of hSP-A to enhance phagocytosis and its natural (in vivo) carbonylation level; 2) in vitro exposure of hSP-A to ozone decreases its phagocytic activity in a dose-dependent manner; 3) in vitro ozone-induced carbonylation reduces the phagocytic activity of SP-A1 and SP-A2 but affects the activity of SP-A2 relatively more than that of SP-A1; 4) at low physiological SP-A concentrations (5 and 10 $\mu\text{g/ml}$ for CHO-cell-expressed and 100 $\mu\text{g/ml}$ for insect-cell-expressed variants), there were no differences between the activities of SP-A1 and SP-A2 after exposure to ozone; however, for proteins not exposed to ozone, SP-A2 enhanced phagocytosis more than SP-A1, as shown previously (38,39); and 5) posttranslational modifications positively affect the overall phagocytic activity of SP-A variants (mammalian cell-expressed SP-As are more active at lower concentrations than insect cell-expressed as we have shown before; Ref. 39). Moreover, posttranslational modifications may modulate the negative impact of ozone on the function of at least the SP-A1 6A⁴ variant (the activity of the insect cell-expressed variant 6A⁴ did not change after ozone exposure conditions under study).

It is well known that air pollutants influence the structure and function of the surfactant system and that this may account for the higher risk of common infections in areas with a high level of air pollution (1). Functional impairment of the host defense molecule SP-A exposed to oxidants, including ozone, may be one of the mechanisms that contribute to the increased risk of hospitalization for pneumonia when ambient ozone levels are high (11,37,47). In fact, in the

present study, we show a significant negative correlation between either the in vivo (in the lung) or in vitro oxidation of hSP-A and its ability to stimulate phagocytosis of *P. aeruginosa* by rat AMs. Thus oxidants in the lung and/or after ozone exposure, in particular, may play a role in the functional impairment of SP-A, and this may, in turn, affect clearance of bacteria from the lung. Moreover, because differences were observed in the level of carbonylation of SP-As isolated from BAL, caution should be exercised to not necessarily consider all SP-As from BAL as functionally equivalent.

SP-A interacts with gram-negative bacteria by binding to the lipid A moiety of LPS, a constituent of the outer membrane (55), whereas for gram-positive bacteria that do not have an outer membrane, it is likely that some of the cell wall components (possibly surface glycoproteins but not lipoteichoic acid and peptidoglycan) are responsible for the bacteria-SP-A interactions (54). For both gram-negative (*P. aeruginosa*) and gram-positive (*S. aureus*) bacteria, ozone-exposed SP-A was significantly less active than nonexposed SP-A. The observed effect points to the possibility that oxidant-induced modifications in SP-A structure may affect its binding to both gram-negative and gram-positive bacteria. Although in vitro studies indicate that ozone exposure can modulate SP-A aggregation (50) and/or oligomerization pattern (56), it is currently unknown whether such changes have an impact on its function in vivo.

In human lungs of healthy adults, the physiological concentration of SP-A in BAL fluid is estimated to be ~0.2–16 mg/l (17). Taking into account the ~100-fold dilution caused by the lavage procedure (17), the lung epithelial lining fluid contains ~20–1,600 µg/ml of SP-A. In some diseases, SP-A concentration in the lung has been reported to be even lower compared with normal levels (26,29). Under normal circumstances, most of the SP-A in the lung is complexed with surfactant lipids, and a relatively small amount of SP-A is “free” (6,51). We (13) speculated before that these low SP-A levels are adequate for innate host defense functions. Using CHO cell-expressed SP-A variants, we found that although both untreated and FA-exposed SP-A2 (1A⁰) exhibited higher activity than those of SP-A1 (6A⁴), the activities of ozone-exposed SP-A1 (6A⁴) and SP-A2 (1A⁰) did not differ at each 5 or 10 µg/ml concentration. This indicates that ozone exposure differentially affects SP-A1 and SP-A2 and, at low concentration, completely abrogates the higher activity observed for SP-A2. Thus the functional differences between SP-A1 and SP-A2 may warrant consideration of both their quantitative and qualitative aspects.

Comparison of phagocytic activities of SP-A1 (1A⁰) and SP-A2 (6A⁴) exposed to FA or ozone indicates that the SP-A2 activity may be affected by ozone more strongly than the SP-A1. However, the data presented do not allow us to determine whether the effect on function is due to an increase in carbonylation or the result of other oxidative modifications (i.e., aggregation, oligomerization, etc.). Tyrosine nitration (63,64) has also been shown to inhibit the binding of carbohydrates and pathogens by SP-A. This action is thought to be due to peroxynitrite production by macrophages. Although this may occur in the lung in response to ozone exposure, it is unlikely to happen under the in vitro ozone exposure conditions used in this study. Taking together these observations, we speculate that individuals who may have more SP-A2 than SP-A1 in their lungs and therefore may originally exhibit higher phagocytic activity and thus be more “protected” from pneumonia may lose their genetic advantage if they live in an area with high ozone pollution.

The data from insect cell-expressed and CHO cell mammalian-expressed SP-As indicate as shown previously (38,39) that, despite significant differences in the posttranslational modifications between the two systems (mammalian and insect), the SP-A2 gene products stimulated phagocytosis more effectively than SP-A1, irrespective of the type of the expression system used. This confirms that the protein backbone is responsible for the gene-specific

differences and that posttranslational modifications positively affect the functional efficiency of SP-A, as a higher concentration is required for insect cell-expressed SP-A variants to obtain results comparable to those of variants expressed in CHO cells (38,39). However, the activity of ozone-exposed SP-A1 and SP-A2 at some concentrations did not differ significantly even though the activity of SP-A2 was significantly higher than that of SP-A1 before ozone exposure. The data presented (Tables 2 and 3) indicate that the impact of posttranslational modifications of SP-A on its function following ozone exposure is likely a minor component.

Of interest, the 6A⁴ variant has been shown to be a risk factor in the pathogenesis of idiopathic pulmonary fibrosis (50) and other lung diseases (12,49). This SP-A variant has a tryptophan instead of arginine (in 1A⁰) at amino acid 219. Although the amino acid tryptophan is more sensitive to ozone exposure than arginine (41), the activity of 1A⁰ was affected more than that of 6A⁴ in response to ozone, indicating that the amino acid context and/or protein structure/folding modulate the susceptibility of amino acid 219 (Trp/Arg) to ozone exposure. Similar results were observed for the 6A⁴/bleomycin synergistic effect on TNF- α production by THP-1 cells after ozone-induced SP-A oxidation (20). Ozone-induced oxidation significantly decreased the ability of 1A, but not of 6A⁴, to stimulate TNF- α production in the presence of bleomycin. Consistent with these findings are the present findings where similar results were observed for the 6A⁴ variant expressed in two different systems, mammalian or insect cells. To further assess whether the differences observed are gene-specific, we investigated the effect of ozone exposure on the ability of another SP-A1 (6A²) variant to enhance phagocytosis and found (as for 6A⁴ variant) that the ozone/FA PI ratio for 6A² was also higher than that for 1A⁰ (SP-A2) at the concentration of 5 μ g/ml (data not shown).

The SP-A1 and SP-A2 gene-specific amino acid differences (at *positions* 66, 73, 81, and 85) are located within the collagen-like region. Recently, we (59) found that Cys⁸⁵ of SP-A1 has a remarkable impact on SP-A structure and function. The gene-specific amino acid differences may affect protein conformation/folding and protein stability (15). This, in turn, may differentially expose certain SP-A1 and SP-A2 amino acids that are more sensitive to ozone oxidation (41) resulting in ozone-induced modification differences between the two gene products. Such differences may differentially affect function and explain the present findings.

In summary: 1) the ability of SP-A to stimulate phagocytosis of *P. aeruginosa* bacteria is reduced by ozone-induced oxidation in a dose-dependent manner; 2) oxidized (carbonylated) SP-A exhibits a decreased ability to enhance phagocytosis of both gram-negative and gram-positive bacteria; 3) the activity of SP-A1 and SP-A2 gene products decreases (depending on concentration) in response to ozone-induced oxidation; and 4) ozone-induced oxidation differentially affects SP-A function with SP-A2 exhibiting a higher decrease in its function than SP-A1. We speculate that functional differences between SP-A1 and SP-A2 gene products exist in vivo and that if individuals live in areas of high air pollution, their SP-A gene-specific activity is differentially affected depending on the protein content of each gene product. Therefore, we further speculate that for patients with relatively low SP-A levels due to a given lung disorder or disease, the effect of air pollution, and particularly of ozone, on SP-A-mediated immune host defense processes should be considered because it may be of critical importance to the overall health status of the lung.

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Fig. 1.

A–C: correlation of the ability of alveolar proteinosis (AP) bronchoalveolar lavage (BAL) human surfactant protein As (hSP-As) to stimulate phagocytosis and their natural (in the lung) oxidation levels. Rat alveolar macrophages (AMs) were mixed with *Pseudomonas aeruginosa* bacteria in the presence or absence (negative control) of SP-A as described in METHODS. After 1-h incubation at 37°C, phagocytosis was assessed. The negative control was set at 100%, and the data are presented as percent of the negative control. The number of independent experiments was 3. Ten SP-A preparations from AP BAL from 6 individuals with different natural (in the lung) oxidation (carbonylation) levels were tested [3 different SP-A preparations from *individual 2* (2-1, 2-2 and 2-3) and *individual 6* (6-1, 6-2, and 6-3) were analyzed]. SP-As were tested for their in vivo carbonylation levels and for their ability to stimulate phagocytosis. SP-A preparations were used at 5 $\mu\text{g}/\text{ml}$. A correlation coefficient was calculated with SigmaStat software at a significance level of $P < 0.05$. **A**: triplicate preparations from *individuals 2* and *6* were analyzed separately. OD, optical density. **B**: results from *individual 2* were pooled together; the same was done for the results from *individual 6*. **C**: OxyBlot Oxidized Protein Detection Kit results for the preparations tested. Prep, preparation.

Fig. 2.

A and **B**: dependence of SP-A-enhanced phagocytosis on the level of in vitro ozone-induced carbonylation of SP-A. The experimental design and the phagocytic index (PI) calculations were performed as described in the legend for Fig. 1 and METHODS. In vitro exposure of SP-A to ozone and detection of the level of protein oxidation were performed as described in METHODS. The correlation coefficient was calculated with SigmaStat software as described in Fig. 1. **A**: the correlation of the ozone-induced carbonyl levels of SP-As with their phagocytic activities. **B**: OxyBlot results for the preparations tested. The SP-A preparation from AP BAL marked in Fig. 1 with asterisk (6-2*) was used for in vitro oxidation with increasing concentrations of ozone: nonexposed (“a”), filtered air (FA)-exposed (“b”), and 0.01 (“c”), 0.1 (“d”), 1 (“e”), and 10 ppm (“f”) of ozone for 4 h. The FA-exposed and untreated (U) SP-As were used as controls. SP-As were tested for their carbonylation levels with the OxyBlot, and their oxidation level is reported as OD multiplied by area. SP-A concentration of 5 µg/ml was used to test the ability of each to stimulate phagocytosis. Data are expressed as means ± SE for 3 independent experiments.

Fig. 3.

Phagocytosis of *P. aeruginosa* or *Staphylococcus aureus* by rat AMs in the presence of in vitro ozone-exposed AP BAL hSP-A is shown. The experimental design and PI calculations were performed as described in the legend for Fig. 1 and METHODS. AP BAL hSP-A was used at a concentration of 5 µg/ml. After in vitro exposure of SP-A to ozone at 1 ppm for 4 h and assessment of the oxidation level, ozone-exposed and untreated SP-As were tested for their ability to enhance phagocytosis. The number of experiments was 3, and the data were considered significant if $P < 0.05$. *Significant differences in the PI values between ozone-exposed and untreated SP-A. OxyBlot data are shown in Fig. 5.

Fig. 4.

A and B: phagocytosis of *P. aeruginosa* by rat AMs in the presence of in vitro ozone-exposed SP-A1 and SP-A2 variants, expressed in mammalian (CHO) cells. The experimental design and PI calculations were performed as described in the legend for Fig. 1 and METHODS. SP-As were exposed to 1 ppm of ozone for 4 h and then tested for their ability to enhance phagocytosis. **A:** ozone-induced carbonyl levels of SP-As tested with Oxy-Blot (*right*). **B:** stimulation of phagocytosis by the SP-As under investigation. O₃, ozone-exposed SP-A. The 1A⁰ and 6A⁴ were used at 5, 10, and 20 µg/ml concentration, and AP BAL hSP-A was used as a positive control at 5 µg/ml. Data are expressed as means ± SE for 3 independent experiments. Differences were considered significant when $P < 0.05$. All SP-As significantly differed from the negative control (without SP-A) except the O₃-exposed 1A⁰ (at 5 µg/ml) and 6A⁴ at the 5 and 10 µg/ml concentrations. The activity of untreated and FA-exposed groups did not differ at any of the concentrations used for 1A⁰, 6A⁴, and hSP-A. “a,” “b,” and “c”: the activity of U, FA, and O₃-exposed 1A⁰ differed from that of 6A⁴, respectively; *significant differences of O₃-exposed SP-A from either U or FA for the same preparation at the same concentration; #significant differences between 20 and 5 µg/ml of 1A⁰ or 6A⁴; ##significant differences between 20 and 10 µg/ml of 1A⁰ or 6A⁴; fnot significant from the negative control (without SP-A).

Fig. 5.

A and *B*: phagocytosis of *P. aeruginosa* by rat AMs in the presence of in vitro ozone-exposed SP-A1 and SP-A2 variants expressed in insect cells. The experimental design and PI calculations were performed as described in the legend for Fig. 1 and METHODS. Insect cell-expressed SP-A variants were used at 100 µg/ml, and hSP-A (a positive control) was used at 5 µg/ml. *A* and *B* are as described in the legend for Fig. 4. The activity of all preparations differed significantly from the negative control (without SP-A). “a”: the activity of untreated 1A differed from that of 6A⁴; *significant differences between untreated and ozone-exposed SP-As in their activity.

Table 1

Comparison of activity between CHO cell-expressed SP-A1 and SP-A2 variants after exposure to FA or to ozone

Concentration of SP-A, µg/ml	Ratio of PI, % 6A ⁴ (SP-A1)-to-1A ⁰ (SP-A2)		
	Untreated SP-A	FA-exposed SP-A	Ozone-exposed SP-A
5	56.09±5.41 *	70.76±3.16 *	107.80±15.19
10	63.05±3.19 *	65.80±0.36 *	85.85±5.09
20	60.73±2.97 *	59.19±1.32 *	68.49±2.69 *

The phagocytic index (PI) values were compared among untreated, filtered air (FA)-exposed, and ozone-exposed surfactant protein A1 (SP-A1) and SP-A2 variants at different concentrations according to the formula of SP-A1-to-SP-A2 (ratio) × 100% ± SE. The number of experiments for each test is $n = 3$ and is shown in Fig. 4.

* $P < 0.05$, significant differences between PIs of SP-A1 and SP-A2.

Table 2

Comparison of activity between ozone-exposed and untreated or FA-exposed SP-A variants expressed in CHO cells

Ratio of PI, %	Concentration of SP-A, µg/ml		
	5	10	20
1A ⁰ (SP-A2)			
Ozone-exposed-to-untreated	45.05±5.07 [*]	59.50±5.22 [*]	63.32 ±2.11 [*]
Ozone-exposed-to-FA- exposed	56.69±6.61 [*]	64.24±1.76 [*]	68.10 ±0.40 [*]
6A ⁴ (SP-A1)			
Ozone-exposed-to-untreated	85.43±9.34 [†]	80.99±7.73 [†]	71.77 ±5.06 ^{*†}
Ozone-exposed-to-FA- exposed	84.56±9.64	84.12±7.56	78.75 ±1.32 ^{*‡}

The ratio of the PI values of ozone-exposed to untreated or FA-exposed SP-A1 and SP-A2 variants at different concentrations is according to the formula of ozone-exposed SP-A-to-FA-exposed (or untreated) SP-A (ratio) × 100% ± SE. The number of experiments for each test is $n = 3$ and is shown in Fig. 4.

^{*} $P < 0.05$, significant differences between PIs compared.

[†] Significant differences between the ratio of 1A⁰ ozone-exposed-to-1A⁰ untreated at 5 µg/ml and each of the ratios of 6A⁴ ozone-exposed-to-6A⁴ untreated at 5, 10, and 20 µg/ml;

[‡] the same as for [†] but for ozone-exposed-to-FA-exposed ratio comparisons between 1A⁰ and 6A⁴.

Table 3

Comparison of activity between ozone-exposed and untreated SP-A variants expressed in insect cells

Ratio of PI	%Differences (mean±SE)
1A (O ₃)-to-1A	62.98±4.99*
6A ⁴ (O ₃)-to-6A ⁴	130.28±13.45 [†]
hSP-A (O ₃)-to-hSP-A	66.23±3.55*
6A ⁴ -to-1A	48.36±4.67*
6A ⁴ (O ₃)-to-1A (O ₃)	98.56±5.78

For phagocytosis of *P. aeruginosa*, rat alveolar macrophages were used. The concentrations of SP-A variants were 100 µg/ml. The positive control (human SP-A; hSP-A) was used at 5 µg/ml concentration. The PI values were compared between ozone-exposed SP-As and their untreated preparations according to the formula of ozone-exposed SP-A-to-untreated SP-A (ratio) × 100% ± SE. The ratio of PI of 6A⁴ to 1A was calculated in the same way, 6A⁴-to-1A (ratio) × 100% ± SE. The number of experiments for each test is $n = 4$ and is shown in Fig. 5.

* $P < 0.05$, significant differences between PIs compared.

[†] $P < 0.05$, significant differences between ratios of 6A⁴ (O₃)-to-6A⁴ and 1A (O₃)-to-1A.