The Effect of Ozone Exposure on the Ability of Human Surfactant Protein A Variants to Stimulate Cytokine Production

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Ozone exposure can cause inflammation and impaired lung function. Human surfactant protein A (SP-A) may play a role in inflammation by modulating cytokine production by macrophages. SP-A is encoded by two genes, *SP-A1* and *SP-A2*, and several allelic variants have been characterized for each gene. These allelic variants differ among themselves in amino acids that may exhibit differential sensitivity to ozone-induced oxidation and this may produce functional differences. We studied the effects of SP-A variants before and after ozone exposure on the production of tumor necrosis factor (TNF)-α and interleukin (IL)-8. These are important proinflammatory cytokines and are expressed by the macrophage-like THP-1 cells. Eight variants were expressed *in vitro*, characterized by gel electrophoresis, and studied. These included six single-gene SP-A alleles and two SP-A variants derived from both genes. Variants were exposed to ozone at 1 ppm for 4 hr at 37°C, and we compared their ability to stimulate cytokine (TNF-α and IL-8) production by THP-1 cells to air-exposed and unexposed SP-A variants. We found that *a*) SP-A2 variants (1A, $1A^0$, $1A^1$) stimulate significantly more TNF-α and IL-8 production than SP-A1 variants (6A, $6A^2$, $6A^4$); *b*) coexpressed SP-A variants ($1A^0/6A^2$, $1A^1/6A^4$) have significantly higher activity than single gene products; *c*) after ozone exposure, all SP-A variants showed a decreased ability to stimulate TNF-α and IL-8 production, and the level of the decrease varied among SP-A variants (26–48%); and *d*) human SP-A from patients with alveolar proteinosis exhibited a minimal decrease (18% and 12%, respectively) in its ability to stimulate TNF-α and IL-8 after *in vitro* ozone exposure. We conclude that biochemical and functional differences exist among SP-A variants, that ozone exposure modulates the ability of SP-A variants to stimulate cytokines by THP-1 cells, and that SP-As from bronchoalveolar lavage (BAL) fluid of certain alveolar proteinosis patients may be oxidized *in vivo*. *Key words*: allele, cytokine, IL-8, ozone, surfactant protein A (SP-A), THP-1 cell, TNF-α. *Environ Health Perspect* 110:79–84 (2002). [Online 18 December 2001] *http://ehpnet1.niehs.nih.gov/docs/2002/110p79-84wang/abstract.html*

Ozone, a major constituent of photochemical air pollution or smog, is a strong oxidizing agent and is potentially toxic. Nearly 113 million people live in U.S. counties with ozone levels above the National Ambient Air Quality (NAAQ) standards of daily exposure limit (0.08 ppm for 8 hours) (*1*). Ozone has very low water solubility and thus cannot effectively penetrate the very thick epithelial lining fluid (ELF) in the upper airways. However, in the lower airways, including bronchiolar and alveolar regions, the ELF is not as thick, thereby allowing ozone to penetrate and react with various constituents in the fluid and the underlying cells. Thus, ozone toxicity is observed mostly in the lower airways. Ozone reacts with unsaturated C=C bonds, amino acids, and other chemical structures, and ozone exposure can lead to edema, inflammation, and epithelial cell damage, contributing to lung injury and pulmonary surfactant derangement (*2*). The components of pulmonary surfactant (a lipoprotein complex) essential for normal lung function are likely to be vulnerable to reaction with inhaled ozone. In fact, both biochemical (*3*) and morphologic alterations (*4,5*) in surfactant have been observed following ozone exposure. Moreover, ozone-exposed surfactant protein A (SP-A) has shown a reduced ability to bind carbohydrates and self-aggregate (*6*) and to inhibit phosphatidylcholine secretion by type II cells (*7*).

Human surfactant protein A consists of two functional genes, *SP-A1* and *SP-A2*, and one pseudogene. More than 30 alleles have been characterized (*8*), and 10 of them appear with > 1% frequency in the general population (*8,9*). These alleles are classified based on nucleotide differences within the coding region, and produce 10 amino acid differences among SP-A1 and SP-A2 alleles. These are located in the signal peptide region, the collagen-like domain, and the carbohydrate recognition domain (CRD) (*9*). Given that there is an order of preference (Cys>Trp=Met>Tyr>His) for ozone to react with free amino acid (*10*) and that certain amino acids in which the SP-A alleles differ may be more susceptible to oxidation, it is possible that certain SP-A alleles are more susceptible to oxidation than others.

Human SP-A, in addition to surfactant related functions, plays a role in innate host defense and the regulation of inflammatory processes in the lung (*11–13*). Human SP-A from bronchoalveolar lavage (BAL) can stimulate cytokine production in the macrophage-like cell line, THP-1, and this effect is inhibited by the surfactant lipids (*14,15*). SP-A variants expressed *in vitro* using the baculovirus-mediated expression system stimulate TNF-α production in THP-1 cells. However, differences were observed between SP-As derived from single and both gene products, and between SP-A1 and SP-A2 variants (*16*).

In the present study, we expressed SP-A variants using mammalian Chinese hamster ovary (CHO)-K1 cells and examined their ability to stimulate cytokine production (TNF-α and IL-8) by THP-1 cells before and after exposure to ozone. We observed biochemical and functional differences among the variants both before and after ozone exposure.

Materials and Methods

Cell lines and cell culture conditions. We used the mammalian CHO-K1 cell line (Cat #CCL 61; American Type Culture Collection, Manassas, VA) for expression of human SP-A variants. The cells were cultured in Glasgow's modified Eagle's medium (GMEM) (Invitrogen, Carlsbad, CA) plus 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2. The stably transfected cell lines from the pEE14-hSP-A transfection were grown in glutamine-free GMEM medium plus 25 μM methionine sulfoximine (MSX); and the stably transfected cell lines from the pCI-neo-hSP-A transfection were maintained in GMEM medium plus 300 mg/L of the antibiotic G418. For the double-gene stably transfected cell lines from the pEE14-hSP-A and pCI-neo-hSP-A transfection, cells were cultured in glutamine-free GMEM medium plus 300 mg/L of G418. The THP-1 cell

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culture and differentiation were performed as described previously (*16*) using RPMI 1640 culture medium with 0.05 mM β-mercaptoethanol and differentiating the cells for 72 hr in the presence of 10^{-8} M vitamin D_3.

Constructs of SP-A alleles. We cloned and expressed SP-A alleles that differ in amino acids that may exhibit differential susceptibility to ozone oxidation, and this process may produce functional differences. The rationale for studying these alleles is based on the functional importance of SP-A and the potential for functional differences among these alleles caused by their amino acid differences. Differences in the ability of these alleles to stimulate proinflammatory cytokine production may explain partly the individual susceptibility to (or protection from) inflammation before and/or after ozone exposure.

For the cloning and expression of SP-A alleles, we used two expression vectors, plasmids pEE14 (LONZA, Berkshire, UK) and pCI-neo (Promega, Madison, WI). pEE14 and pCI-neo contain different selection markers, neomycin phosphotransferase and glutamine synthetase, respectively. The use of two different selection markers facilitated selection efforts when screening for the transgenic cell lines that expressed both the *SP-A1* and *SP-A2* genes. For the *pEE14-hSP-A* construct, a 1.3 kb cDNA segment from an *SP-A2* allele (e.g., *1A*, $1A^0$, $1A^1$) was cloned into the *Sma*I restriction site of the pEE14 vector. This 1.3 kb insert cDNA includes 0.74 kb coding region, about 0.1 kb of 5′UTR, and 0.5 kb of 3′UTR. Expression of the SP-A cDNA is controlled by the human cytomegalovirus (CMV) immediate-early enhancer/promoter region. Stable transgenic cells are selected in glutamine-deficient medium (*17*).

For the *pCI-neo-hSP-A* construct, a 1.3 kb cDNA segment from an *SP-A1* allele (e.g. $6A^2$, $6A^4$) was inserted into Xho I and Acc I of the pCI-neo vector. This 1.3 kb insert cDNA includes 0.74 kb of coding region, about 0.1 kb 5′UTR, and 0.5 kb 3′UTR. A 0.9 kb cDNA segment of the *SP-A1* allele *6A* was cloned into *Eco* RI site of the pCI-neo vector. The 6A insert, in addition to the coding region, includes about 0.1 kb 5′UTR and 0.1 kb 3′UTR. The expression of cDNA in the PCI-neo vector is also driven by the human CMV immediate-early enhancer/promoter region. The intron sequence between the promoter region and the insert DNA in the vector can increase the expression level (*18*). Stably transfected cells were selected for neomycin phosphotransferase activity (neomycin resistance). Recombinant constructs were made according to standard methods (*19*).

CHO cell transfection and selection of stably transfected cell lines. CHO-K1 cells were cultured in GMEM. Adherent CHO K1 cells were transfected with *pCI-neo-hSP-A*, *pEE14-hSP-A*, *pCI-neo*, pEE14, or mock-transfected with LipofectAMINE (GIBCO/BRL) according to the manufacturer's instructions. Approximately, 5×10^6 cells in one 60-mm plate were transfected with 8 μg of plasmid DNA in GMEM in the presence of 33 μL of LipofectAMINE and in the absence of serum and antibiotics. Incubations were done for periods of 6–22 hr. The transfected cells were then incubated in GMEM containing 10% FBS for 48 hr. The cells were split at 1:8 and incubated for 48 hr in glutamine-free GMEM containing 10% dialyzed FBS supplemented with 25 μM MSX. CHO cells from pCI-neo-hSP-A and pCI-neo transfection were cultured in GMEM plus 10% FBS, and 600 mg/L of G418. After 2–3 weeks, we isolated colonies of vigorously proliferating cells from transfected cultures using a cloning cylinder. After the cells reached confluence, fresh culture medium was applied. We then screened primary cell clones using a semiquantitative solid-phase immunoassay and by immunoblotting with rabbit anti-SP-A antibody (*16*). Selected cell lines of transformants were further amplified after exposure to a higher concentration of MSX (60 μM). For double transfections with *SP-A1* and *SP-A2*, we selected and screened clones as described above, except that the selection medium was glutamine-free and contained the selection factor 600 mg/L G418. Three *SP-A1* alleles (i.e., *6A*, $6A^2$, $6A^4$) were cloned into expression plasmid pCI-neo, three *SP-A2* alleles (i.e., *1A*, $1A^0$, $1A^1$) were cloned into expression plasmid pEE14, and two co-expression combinations, $1A^0/6A^2$, $1A^1/6A^4$ were produced by cotransfection of both plasmids.

After selection, more than 40 stably transfected cell clones were obtained. We verified SP-A expression in single gene transfected clones using Western blotting. To identify the expression of both genes of *SP-A1* and *SP-A2*, we isolated mRNA from putative clones that can grow in the presence of the antibiotic G418 and in glutamine-deficient medium. We used *SP-A1* and *SP-A2* specific primers for gene-specific RT-PCR to verify expression of both genes, and we used PCR-cRFLP genotyping (*8*) to confirm each cloned allele. Clones that expressed both *SP-A1* and *SP-A2* genes were propagated. Expression of *SP-A1* and *SP-A2* genes was further verified by two-dimensional gel electrophoresis and Western blot analysis. About 80% of the clones transfected with *pEE14-hSP-A* expressed SP-A, about 30–50% of the clones transfected with *pCI-neo-hSP-A* expressed SP-A, and only 10–30% of clones transfected with both *SP-A1* and *SP-A2* constructs expressed both *SP-A1* and *SP-A2* proteins.

We detected SP-A protein in the medium of the transfected cells, but no SP-A protein in the medium of mock transfected cells (data not shown), indicating that CHO-K1 cells express only transfected human SP-A gene(s) rather than their own SP-A gene.

Preparation of SP-A variants and native human SP-A. We recovered and purified SP-A from the culture medium using mannose-affinity chromatography according to the method of Fornstedt and Porath (*20*). Briefly, we harvested about 200 mL of serum-free culture medium (2×10^7 cells/plate/10 mL) from a 5-day culture. We removed CHO cells and cell fragments from the culture medium by centrifugation at $1{,}000 \times g$ for 10 min at 4°C. The supernatant was then transferred to new tubes and passed through a sepharose-6B column followed by a polymyxin-B-agarose column (all columns from Sigma, St. Louis, MO). The solution containing SP-A was adjusted to 10 mM Ca^{2+} with 1 M $CaCl_2$, and was passed through a mannose-sepharose 6B column (40 mL) at about 60 mL/hr. The column was then washed with 200 mL of 5 mM Tris, 1 mM $CaCl_2$, pH 7.5. SP-A was eluted with an elution buffer containing 5 mM Tris, 2 mM EDTA, pH 7.5 and collected in 2 mL fractions. We purified SP-A by repeating the mannose affinity procedure and gel filtration with a Superose 6 column (Amersham Pharmacia Biotech, Piscataway, NJ). SP-A was dialyzed as described (*16*). The native human SP-A was purified from BAL fluid obtained from alveolar proteinosis patients by a method previously described (*16*). SP-A was concentrated using Amicon Centriprep-10 concentrators (Amicon, Beverly, MD). All procedures were performed at 4°C or on ice. We determined protein concentration using the Micro-BCA method of Smith et al. (*21*) (Pierce, Rockford, IL) with RNase A as standard. SP-A was aliquoted and stored at –70°C. Gel electrophoresis, silver staining, and Western blotting of SP-A were done as described previously (*16*).

As a part of the SP-A purification, we removed lipopolysaccharide (LPS) from SP-A preparations by incubating the purified SP-A with polymyxin-B-agarose (1 mL agarose beads/mg SP-A) according to manufacturer's instructions (Sigma, St. Louis, MO). We assessed the LPS content of SP-A preparations using the QCL-1000 assay, a test employing Limulus Amebocyte Lysate (BioWhitaker, Walkersville, MD). Purified preparations of SP-A contained less than 0.1 pg of LPS/μg of SP-A.

Exposure of SP-A to ozone. We exposed SP-A proteins at a concentration of 1 mg/mL to ozone in 24-well tissue culture plates as described previously (*22*). Each well

contained 100 µL of solution, which was about 0.5 mm deep. Plates were weighed before and after each exposure to check for changes in sample volumes. We conducted ozone concentration and time course exposures to determine optimal exposure conditions. An ozone concentration of 1 ppm for 4 hr was optimal for protein oxidation, and all of the following exposures were made using these conditions. After exposure, proteins were transferred to sterile microcentrifuge tubes and stored at –80°C until further use. At least three independent experiments were performed in this study.

Detection of protein oxidation. We detected protein oxidation using the OxyBlot oxidized protein detection kit (Intergen, Purchase, NY) (*22*). Briefly, after ozone exposure, samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH). DNPH-derivatized protein (200 ng) was blotted onto nitrocellulose and immunodetection of oxidized proteins was done with rabbit anti-DNPH and goat anti-rabbit IgG (HRP-conjugated) antibodies. Blots were exposed to XAR film following enhanced chemiluminescent detection.

SP-A treatment of THP-1 cells and ELISA assay for TNF-α and IL-8. After differentiation with 10^{-8} M vitamin D_3 for 72 hr, THP-1 cells were washed with culture medium and exposed to SP-A (50 µg/mL). As positive controls, we used human SP-A from BAL fluid (50 µg/mL) and LPS (0.1 ng/mL). Incubations were performed with 2×10^6 cells/mL in 24-well culture plates for periods ranging from 0 to 12 hr or using a 4-hr time point. Fetal calf serum (10%) was present in all incubations.

We performed TNF-α and IL-8 ELISA assays as recommended by the manufacturer (PerSeptive Diagnostics, Cambridge, MA). We analyzed samples with 100 µL/well in a 96-well plate. And we obtained a reference curve by plotting the TNF-α or IL-8 concentration of several dilutions of standard protein versus absorbance.

Statistical methods. TNF-α and IL-8 data obtained from three independent experiments were expressed as the mean ± SE. Levels of TNF-α and IL-8 induced by various SP-As were compared and analyzed by multiple comparison ANOVA test. Differences were considered significant when the p value was < 0.05.

Results

Characteristics of SP-A variants from CHO-K1 cells. Six SP-A alleles *6A*, *6A*^{2}, *6A*^{4}, *1A*, *1A*^{0}, *1A*^{1}, and the two combinations *1A*^{0}/*6A*^{2} and *1A*^{1}/*6A*^{4} were successfully expressed as described above. Expressed SP-A protein was secreted into the culture medium, suggesting that the signal peptide of human SP-A is functional in CHO-K1 cells, and the secreted SP-A was purified. We used serum-free GMEM medium for SP-A expression because it was difficult to purify SP-A from serum-containing medium. After purification according to the method described in "Materials and Methods," SP-A purity was assessed at 99% (Figure 1). The SP-A pattern shown in Figure 1 indicates that the *in vitro* expressed SP-A from mammalian CHO-K1 cells is similar to SP-A from human BAL fluid. This indicates that similar post-translational modifications occur in the *in vitro* expressed SP-A protein and the native human SP-A protein. These post-translational modifications may be important for some SP-A functions.

Oxidation of SP-A after ozone exposure. We studied two representative SP-A alleles—SP-A1 allele 6A and SP-A2 allele 1A—in ozone time-course and dose–response experiments, to determine the optimal ozone exposure time and concentration. Alleles 1A and 6A were exposed to ozone at 1 ppm for 30 min, 1 hr, 2 hr, 4 hr, and 12 hr. The results showed that neither 1A nor 6A is oxidized before ozone exposure, but both are oxidized after ozone exposure (Figure 2A). The degree of SP-A oxidation increased as the length of exposure time (from 30 min to 4 hr) increased. When the exposure was prolonged to 12 hr, we saw no marked difference between the 4-hr exposure and the 12-hr exposure. For the ozone concentration course experiment, we exposed SP-A alleles 1A and 6A for 4 hr at the following concentrations of ozone: 0.1 ppm, 0.5 ppm, 1 ppm, and 2 ppm. Figure 2B shows that the signal for oxidized SP-A increased from 0.1 ppm to 1 ppm, but no marked difference was detected between 1 ppm and 2 ppm.

Subsequently, we determined the functional changes of SP-A following ozone exposure. We tested ozone-exposed SP-A alleles 1A and 6A from the ozone time-course and ozone dose–response experiments for their ability to stimulate TNF-α production by THP-1 cells (Figure 3). Before ozone treatment, the SP-A2 allele 1A and the SP-A1 allele 6A stimulate TNF-α production (814 ± 47 pg/mL and 452 ± 35 pg/mL, respectively, vs. positive control hSP-A 437 ± 21pg/mL) by THP-1 cells. After ozone exposure, the ability of SP-A alleles 1A and 6A to stimulate TNF-α production decreased. When SP-A was exposed to ozone from 30 min to 4 hr and then used to stimulate THP-1 cells, the degree of stimulation of the TNF-α level consistently decreased as the ozone exposure time of the SP-A increased. The TNF-α levels resulting from treatment with 1A and 6A at 4 hr are 66% and 60%, respectively, of the levels observed before ozone treatment. However, no marked difference of TNF-α levels exists

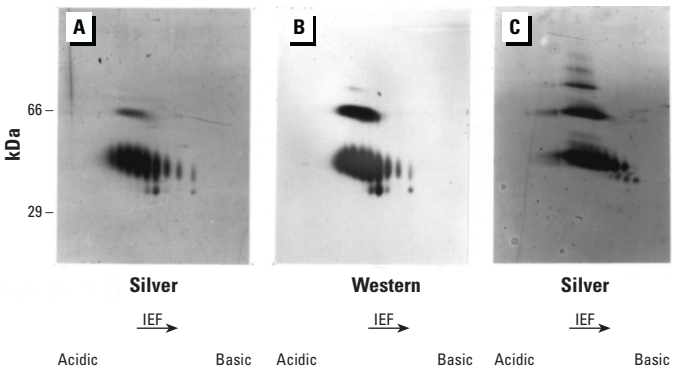

Figure 1. Gel electrophoresis of SP-A from *in vitro*-expressed and BAL-derived SP-A. IEF, isoelectric focusing. (*A*) and (*B*) depict CHO-K1-expressed SP-As and (*C*) depicts SP-A from BAL of an alveolar proteinosis patient. Purified SP-A was separated by two-dimensional gel electrophoresis. Gels were subjected to silver staining (*A*,*C*) and Western blotting with an antiserum to human SP-A (*B*). The numbers on left indicate molecular mass.

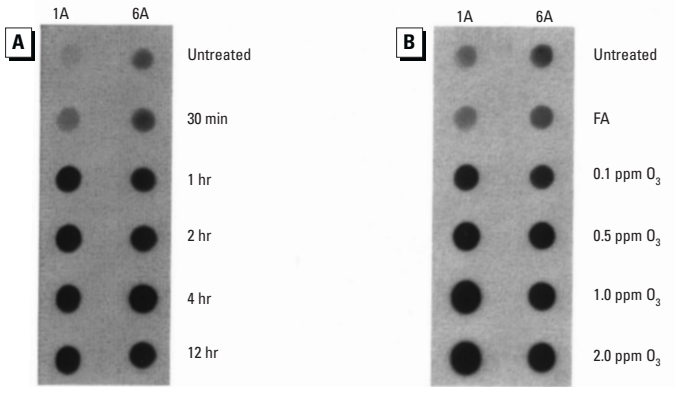

Figure 2. SP-A oxidation as a function of time and ozone concentration. FA, filtered air. Two *in vitro*-expressed SP-A variants, 1A and 6A, from SP-A2 and SP-A1, respectively, were exposed to ozone (1 ppm) at different times (*A*) and at different ozone concentrations for 4 hr (*B*). Each sample (200 ng) was blotted onto a membrane and the oxidized proteins were detected using the OxyBlot method. The optimal time and ozone concentration for exposure was 4 hr and 1.0 ppm.

between the 4 hr exposure time point and the 12 hr time point, suggesting that SP-A oxidation after a 4 hr exposure at 1 ppm has reached a plateau with regard to the end point studied here.

We next assessed the effect of ozone concentration on SP-A oxidation and function. We exposed SP-A alleles 1A and 6A to ozone at different concentrations including 0.1 ppm, 0.5 ppm, 1 ppm, and 2 ppm for 4 hr. Figure 4 shows that after exposure to 1 ppm ozone, 1A and 6A stimulate TNF-α by THP-1 cells at significantly lower levels than SP-A exposed to 0.1 ppm or 0.5 ppm ($p < 0.05$). No significant difference of TNF-α levels was found when exposure was increased from 1 ppm to 2 ppm.

Given SP-A oxidation and functional changes, we concluded that exposure to 1 ppm ozone for 4 hr is the optimal condition, so we used this standard for subsequent experiments.

Comparison of the effects of unexposed and ozone-exposed SP-A on the stimulation of cytokines by THP-1 cells as a function of time. Vitamin D_3-differentiated THP-1 cells were stimulated for different time periods (1–12 hr) with either normal (unexposed) or ozone-exposed SP-A1 allele 6A and SP-A2 allele 1A (50 μg/mL). Figure 5 shows that after 1 hr of incubation with SP-A, TNF-α was detected in the culture medium of THP-1 cells at levels above background. The content of TNF-α in the medium increased dramatically from 1 to 4 hr, and reached a peak at 4 hr. The content of TNF-α subsequently decreased, although it was still above background at 12 hr. The levels of TNF-α stimulation by unexposed SP-A 6A and 1A were significantly higher than those from ozone-exposed SP-A 6A and 1A. However, the time course of TNF-α production was identical for the unexposed and ozone-exposed variants.

Similarly, we analyzed IL-8 production by THP-1 cells in response to SP-A in a time course experiment. The results are shown in Figure 6. IL-8 was detected in the medium after a 2-hr incubation. The level of IL-8 dramatically increased from 2 to 4 hr, and continued to increase from 4 to 12 hr. At the 4-hr time point the level of IL-8 reached about 80% of the highest level measured (12-hr time point). Therefore, we considered a 4-hr time point optimal for measuring both TNF-α and IL-8 to compare the effects of different SP-A variants in subsequent experiments.

Comparison of ozone-exposed, air-exposed, and unexposed SP-A variants on the stimulation of TNF-α production. We tested eight SP-A variants for their ability to stimulate TNF-α production. Of these, three were SP-A1 alleles (6A, $6A^2$, $6A^4$), three were SP-A2 alleles (1A, $1A^0$, $1A^1$), and two were coexpressed SP-As ($1A^0/6A^2$, $1A^1/6A^4$). We performed three independent experiments involving treatment of THP-1 cells with SP-A at a concentration of 50 μg/mL for 4 hr. The content of TNF-α in THP-1 cell culture supernatants after stimulation with SP-A is summarized in Table 1. All six SP-A alleles and the two coexpressed SP-As stimulate TNF-α production by THP-1 cells. The SP-A2 variants 1A, $1A^0$, $1A^1$ stimulate TNF-α production significantly more than SP-A1 variants 6A, $6A^2$, $6A^4$ ($p < 0.01$). Both coexpressed SP-A variants have significantly higher activity than SP-A1 variants 6A, $6A^2$, $6A^4$ ($p < 0.01$), and they also appear to have higher activity than SP-A2 variants 1A, $1A^0$, $1A^1$. The ability of air-exposed SP-A variants to stimulate TNF-α production was similar to that of unexposed SP-A variants. As positive controls, we used human SP-A from BAL fluid (50 μg/mL) and LPS (0.1 ng/mL), and both stimulated TNF-α production to levels of 437 ± 21 pg TNF-α/mL and 384 ± 23 pg TNF-α/mL, respectively. In untreated negative controls, the level of TNF-α in the medium was 26 ± 7 pg TNF-α/mL (Table 1).

After ozone exposure, all the SP-A variants were significantly inhibited in their ability (range 33–48%) to stimulate TNF-α production compared with air-exposed SP-A variants, but the percent change (O_3/air) for SP-A variants $6A^4$ and $1A^1/6A^4$ was larger than that of other variants. For $6A^4$ there was a 55% change versus 64% (6A) or 67% ($6A^2$) and for $1A^1/6A^4$, 52% versus 61% for $1A^0/6A^2$. These observations indicate that the oxidation of SP-A decreases its ability to stimulate TNF-α production, and that differences may occur among SP-A variants. Human SP-A from BAL of an alveolar proteinosis patient was used as a positive control. The ability of this ozone-exposed SP-A to stimulate TNF-α production was reduced by only 18% compared to that of the air-exposed SP-A.

Comparison of ozone-exposed, air-exposed, and unexposed SP-A variants on the stimulation of IL-8 production. We further analyzed cytokine IL-8 content in the medium of THP-1 cells after stimulation with SP-A variants for 4 hr. The results are summarized in Table 2. All of the SP-A variants 6A, $6A^2$, $6A^4$, 1A, $1A^0$, $1A^1$, $1A^0/6A^2$, and $1A^1/6A^4$ stimulate IL-8 production by THP-1 cells, and the activity of air-exposed SP-A variants was similar to that of unexposed SP-A variants. The levels of IL-8 for all treatments are substantially higher than that of the negative control. The SP-A2 variants 1A, $1A^0$, $1A^1$ have significantly higher activity than SP-A1 variants 6A, $6A^2$, $6A^4$. Coexpressed SP-As $1A^0/6A^2$, $1A^1/6A^4$ consisting of both gene products have significantly higher activity than SP-A1 variants 6A, $6A^2$, $6A^4$ ($p < 0.01$) and SP-A2 variants 1A, $1A^0$, $1A^1$ ($p < 0.05$).

After ozone exposure, all of the SP-A variants had a significantly reduced ability (range 26–45%) to stimulate IL-8 production compared to that with air-exposed SP-A variants ($p < 0.01$). The percent change

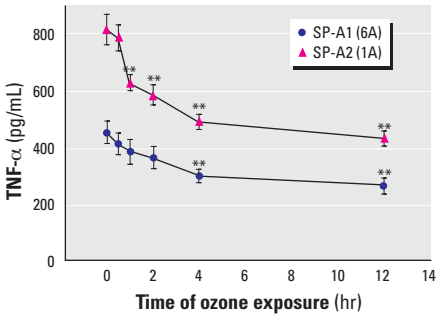

Figure 3. Time course of the effect of ozone-exposed SP-A on TNF-α production. Data were obtained from three independent experiments.
**$p < 0.01$; there is a significant difference between ozone-exposed SP-A and untreated SP-A (zero hour point).

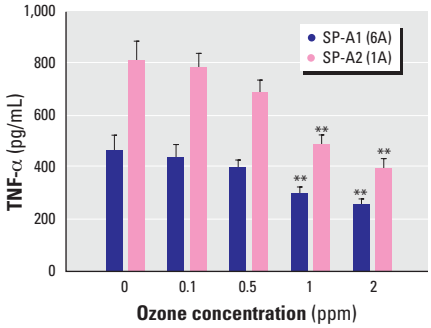

Figure 4. Ozone dose response study of the ability of SP-A to stimulate TNF-α production. Data were obtained from three independent experiments.
**$p < 0.01$; there is a significant difference between ozone-exposed SP-A and untreated SP-A (zero ppm ozone exposure).

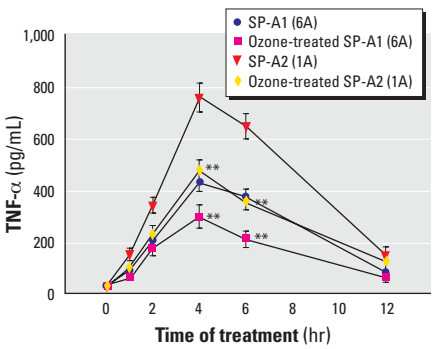

Figure 5. Time course of TNF-α production by THP-1 cells treated with SP-A before and after ozone exposure. Data were obtained from three independent experiments.
**$p < 0.01$; there is a significant difference between ozone-exposed SP-A (1A or 6A) and untreated SP-A (1A or 6A).

(O_3/air) for $6A^4$ and $1A^1/6A^4$ variants tended to be lower than that for other SP-A variants (Table 2). The activity of the ozone-exposed SP-A from BAL fluid of the alveolar proteinosis patient (described previously) was only 13% lower than for the air-exposed SP-A.

Discussion

Ozone, a strong oxidative agent, can impair lung function and cause lung injury and inflammation via oxidative reactions with epithelial cells and surfactant components in the lung and airways. A remarkably high variability in response to ozone has been observed (*23–25*), and genetic factors have been implicated in this variability. Human SP-A plays a role in inflammation by regulating cytokine production by macrophages. SP-A is encoded by two genes, *SP-A1* and *SP-A2*, and several allelic variants have been characterized for each gene. These allelic variants differ among themselves in amino acids that may exhibit differential susceptibility to ozone exposure and this may result in functional differences. Differences in the ability of these alleles to stimulate proinflammatory cytokine production may explain in part the individual susceptibility to inflammation before and/or after ozone exposure. In this study, we investigated the hypotheses that biochemical and functional differences exist among SP-A variants and that ozone affects the functional abilities of these protein variants. We observed differences in function among SP-A1, SP-A2, and coexpressed SP-A variants, before and after ozone exposure. All of the ozone-exposed SP-A variants exhibited a decreased ability to stimulate cytokine production, although the degree of the decrease varied among SP-A variants, with allele $6A^4$ and the coexpressed $1A^1/6A^4$ showing the greatest decrease. This variability may reflect differences among individuals in their susceptibility to ozone, and may be, in part, the basis for individual variability in susceptibility to lung disease.

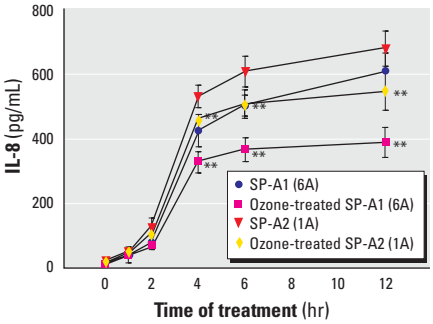

Figure 6. Time course of IL-8 production by THP-1 cells treated with the SP-A before and after ozone exposure. Data were obtained from three independent experiments.
**$p < 0.01$; there is a significant difference between ozone-exposed SP-A (1A or 6A) and untreated SP-A (1A or 6A).

Native SP-A is expressed by several cell types, including the type II alveolar epithelial cells in the lung, and secreted into the lung alveolar space (*26*). As the SP-A precursor is processed to form mature SP-A, it undergoes several post-translational modifications including *N*-linked glycosylation, hydroxylation of proline residues, addition of sialic acid to the oligosaccharide, and signal peptide cleavage (*26,27*). These post-translational modifications may be involved in some SP-A functions (*28*). Of interest, *in vitro* expressed SP-As from the baculovirus-mediated insect cell expression system, which are deficient in proline hydroxylation and show incomplete *N*-linked glycosylation (*16,29*), exhibit a lower activity in their ability to stimulate TNF-α production by THP-1 cells than does SP-A expressed from mammalian (CHO) cells, which undergoes proline hydroxylation and more complete processing of *N*-linked oligosaccharides. *In vitro* expressed SP-As from CHO cells appear to have post-translational modifications similar to those on native human SP-A, so these SP-A preparations should have function(s) similar to those of native human SP-A. Moreover, SP-A expressed from transfected CHO cells is not oxidized (as SP-A from alveolar proteinosis patients might be) before ozone exposure, which is an essential prerequisite for this study. The quality of SP-A samples prepared from BAL fluid of alveolar proteinosis patients (an enriched source for SP-A) varied, with two out of three tested being highly oxidized (*22*) before ozone exposure.

The production of both TNF-α and IL-8 by THP-1 cells was enhanced after stimulation by SP-A, but the kinetics of the changes and pattern profiles of each cytokine differed, suggesting complex interactions. TNF-α was detected in the medium after 1 hr of SP-A stimulation, and peaked at the 4-hr time point. IL-8 was detected in the medium at 2 hr after stimulation with SP-A. The content of IL-8 in the medium continued to increase from 2 hr to 12 hr. Although the IL-8 production is likely to be induced by both SP-A (*15*) and TNF-α (*30*), the relative contributions of SP-A and TNF-α to the increase in IL-8 production are unknown and will be the focus of further investigation. The most likely reason why this proteinosis-derived SP-A preparation shows a very small change in its functional capability after *in vitro* ozone exposure is probably that it was already highly oxidized before *in vitro* ozone exposure (*22*); hence, the low percent change after ozone exposure.

Although all human SP-A variants expressed in CHO cells stimulate TNF-α

Table 1. TNF-α (pg/mL) production by THP-1 cells after a 4-hr stimulation by SP-A[a] before or after its exposure to ozone.[b]

SP-A variants or control	Unexposed SP-A	Exposed SP-A		
		+ Air	+ Ozone (1 ppm)	O_3/air (%)
6A	479 ± 24	495 ± 21	321 ± 19**	64
$6A^2$	387 ± 15	406 ± 26	274 ± 14**	67
$6A^4$	456 ± 32	489 ± 28	267 ± 23**	55
1A	824 ± 41	794 ± 35	521 ± 24**	65
$1A^0$	756 ± 33	801 ± 41	472 ± 38**	59
$1A^1$	713 ± 49	782 ± 31	493 ± 23**	63
$1A^0/6A^2$	984 ± 54	951 ± 45	580 ± 33**	61
$1A^1/6A^4$	923 ± 43	907 ± 23	471 ± 23**	52
hSP-A	437 ± 21	395 ± 31	324 ± 19*	82
LPS (0.1 ng/mL)	384 ± 23			
Untreated control	26 ± 7			

[a]SP-A, 50 μg/mL. [b]Ozone, 1 ppm for 4 hr at 37°C. *$p < 0.05$, **$p < 0.01$ ozone-exposed SP-A versus air-exposed SP-A.

Table 2. IL-8 (pg/mL) production by THP-1 cells after a 4-hr stimulation by SP-A[a] before or after its exposure to ozone.[b]

SP-A variants or control	Unexposed SP-A	Exposed SP-A		
		+ Air	+ Ozone (1 ppm)	O_3/air (%)
6A	478 ± 39	461 ± 35	312 ± 32 **	68
$6A^2$	439 ± 45	428 ± 27	273 ± 24 **	64
$6A^4$	468 ± 38	435 ± 32	243 ± 38 **	56
1A	564 ± 41	578 ± 43	433 ± 34 **	74
$1A^0$	621 ± 48	604 ± 41	419 ± 38 **	69
$1A^1$	603 ± 39	569 ± 34	375 ± 39 **	65
$1A^0/6A^2$	728 ± 45	685 ± 32	418 ± 38 **	61
$1A^1/6A^4$	789 ± 19	768 ± 46	422 ± 32 **	55
hSP-A	382 ± 41	369 ± 32	321 ± 27	87
LPS (0.1 ng/mL)	452 ± 39			
Untreated control	69 ± 31			

[a]SP-A, 50 μg/mL; [b]Ozone, 1 ppm for 4 hrs at 37°C. **$p < 0.01$, ozone-exposed SP-A versus air-exposed SP-A.

production by THP-1 cells, as shown for SP-A variants expressed by insect cells (*16*), the activity is higher in SP-A variants expressed by mammalian cells. The relative changes observed among variants produced either by mammalian or insect cells are similar. In both cases, SP-A2 alleles have higher activity than SP-A1 alleles, and SP-As derived from both genes have higher activity than SP-As of single gene products. All of the SP-A2 alleles differ from all of the SP-A1 alleles at four amino acids within the collagen-like domain, and these differences may affect SP-A structure and function. After ozone exposure, all of the human SP-As had decreased ability to stimulate cytokine production by THP-1 cells, but some SP-A variants showed a greater decrease than others. In particular, allele $6A^4$ and coexpressed $1A^1/6A^4$ showed a greater decrease than other variants. Allele $6A^4$ has a tryptophan at position 219, whereas all other alleles have an arginine (*9*). Tryptophan is more susceptible than arginine to oxidation by ozone (*10*). Allele $1A^1$ has a lysine at position 223, and the other alleles have a glutamine (*9*). Although neither lysine nor glutamine is sensitive to oxidation, these differences in amino acids may have an indirect effect on protein conformation, which in turn may expose amino acids that are more sensitive to ozone oxidation. In fact, oxidized SP-A has been shown to exhibit changes in its molecular conformation and a decreased ability to interact with alveolar macrophages (*7*). Similarly, ozone-exposed human SP-A variants might undergo changes in their conformation that may decrease their ability to interact with THP-1 cells. It is not known how SP-A exerts its activity on THP-1 cells, although it is known that it can bind to C1q receptor (*31*). Altered SP-A conformation may lead to changes in such an interaction.

In summary, our results indicate that *a*) single SP-A gene variants and coexpressed (both SP-A gene products) variants can be successfully expressed and purified from stably transfected mammalian cell lines; *b*) the *in vitro* expressed variants can stimulate cytokine production (TNF-α, IL-8) in a time- and dose-dependent manner; *c*) differences exist among SP-A variants in the degree of stimulation of cytokine production; *d*) the time- and dose-dependent pattern of stimulation is maintained after ozone exposure of the SP-A variants, but the degree of stimulation is decreased; and *e*) the percent change in the degree of stimulation of cytokine production by SP-A variants before and after ozone exposure varies among certain variants. It is possible that the SP-A of certain individuals is more susceptible to ozone oxidation than others, and this may help explain individual differences in susceptibility or resistance to ozone-related pulmonary disease.

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