

Pulmonary surfactant inhibits LPS-induced nitric oxide production by alveolar macrophages

P. R. MILES,^{1,2} L. BOWMAN,¹ K. M. K. RAO,¹ J. E. BAATZ,³ AND L. HUFFMAN^{1,2}

¹Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown 26505; ²Department of Physiology, West Virginia University School of Medicine, Morgantown, West Virginia 26506; and ³Department of Pediatrics, Medical University of South Carolina, Charleston, South Carolina 29425-3313

Miles, P. R., L. Bowman, K. M. K. Rao, J. E. Baatz, and L. Huffman. Pulmonary surfactant inhibits LPS-induced nitric oxide production by alveolar macrophages. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L186–L196, 1999.—The objectives of this investigation were 1) to report that pulmonary surfactant inhibits lipopolysaccharide (LPS)-induced nitric oxide ($\cdot$ NO) production by rat alveolar macrophages, 2) to study possible mechanisms for this effect, and 3) to determine which surfactant component(s) is responsible. $\cdot$ NO produced by the cells in response to LPS is due to an inducible $\cdot$ NO synthase (iNOS). Surfactant inhibits LPS-induced $\cdot$ NO formation in a concentration-dependent manner; $\cdot$ NO production is inhibited by $\sim$ 50 and $\sim$ 75% at surfactant levels of 100 and 200 μ g phospholipid/ml, respectively. The inhibition is not due to surfactant interference with the interaction of LPS with the cells or to disruption of the formation of iNOS mRNA. Also, surfactant does not seem to reduce $\cdot$ NO formation by directly affecting iNOS activity or by acting as an antioxidant or radical scavenger. However, in the presence of surfactant, there is an $\sim$ 80% reduction in the amount of LPS-induced iNOS protein in the cells. LPS-induced $\cdot$ NO production is inhibited by Survanta, a surfactant preparation used in replacement therapy, as well as by natural surfactant. $\cdot$ NO formation is not affected by the major lipid components of surfactant or by two surfactant-associated proteins, surfactant protein (SP) A or SP-C. However, the hydrophobic SP-B inhibits $\cdot$ NO formation in a concentration-dependent manner; $\cdot$ NO production is inhibited by $\sim$ 50 and $\sim$ 90% at SP-B levels of 1–2 and 10 μ g/ml, respectively. These results show that lung surfactant inhibits LPS-induced $\cdot$ NO production by alveolar macrophages, that the effect is due to a reduction in iNOS protein levels, and that the surfactant component responsible for the reduction is SP-B.

lipopolysaccharide; surfactant protein B; hydrophobic surfactant proteins; inducible nitric oxide synthase

NITRIC OXIDE ($\cdot$ NO) is a free radical that is produced by a variety of cell types in the lungs. The synthesis of $\cdot$ NO from L-arginine is catalyzed by a family of enzymes known as $\cdot$ NO synthases (NOSs). One very important isoform of this enzyme is inducible NOS (iNOS). It has been shown that $\cdot$ NO produced by iNOS plays an important role in defense against airborne pathogens and is involved in tissue damage associated with inflammatory processes in the lungs (11, 17). Upregulation of iNOS is transcriptionally regulated and can occur after

exposures to inflammatory stimuli such as cytokines and/or endotoxin (11). Examples of some lung cells that can generate $\cdot$ NO by means of iNOS include alveolar type II cells (14), lung fibroblasts (18), pulmonary arterial smooth muscle cells (31), and neutrophils (43).

Alveolar macrophages are mobile phagocytic cells located within the alveolar regions and small airways of the lungs. These cells represent a primary line of defense against the adverse effects of inhalation of bacteria and foreign particles. It is well known that alveolar macrophages can be induced to produce $\cdot$ NO via iNOS. For example, some investigators (21, 33) have demonstrated that rat alveolar macrophages produce $\cdot$ NO in response to inflammatory stimuli or cytokines, e.g., lipopolysaccharide (LPS) or interferon- γ (IFN- γ). These inflammatory stimuli act by causing expression of iNOS mRNA (22, 24). In fact, one of the major sources of $\cdot$ NO produced by iNOS in the lungs during the inflammatory process is the alveolar macrophage.

Pulmonary surfactant is a complex mixture of phospholipids (PLs), lipids, and proteins that lines the alveolar regions of the lungs. Although its major function is to prevent alveolar collapse by lowering surface tension forces, lung surfactant is also known to have some effects on alveolar macrophages. For example, Thomassen et al. (39, 40) have shown that in vitro exposure of human alveolar macrophages to two surfactant preparations used for replacement therapy, Exosurf and Survanta, results in suppression of endotoxin-induced production of cytokines, i.e., tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6. Other investigators have shown that one of the major surfactant proteins, surfactant protein (SP) A, has effects on alveolar macrophages, such as inhibiting the release of TNF- α from LPS-stimulated cells (25), enhancing phagocytosis (41), or stimulating the secretion of granulocyte-macrophage colony-stimulating factor from the cells (4). These results demonstrated that lung surfactant can affect alveolar macrophage function. In addition, surfactant is part of the normal environment for these cells in vivo. Because alveolar macrophages represent a major source of $\cdot$ NO production during inflammatory processes and because surfactant can affect some responses of these cells, we wondered what effects surfactant has on cellular $\cdot$ NO formation. Therefore, the objectives of this investigation were 1) to determine the effects of rat lung surfactant on $\cdot$ NO production by LPS-stimulated rat alveolar macrophages, 2) to study some possible mechanisms for these effects, and 3) to

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determine which surfactant component(s) is responsible for the effects.

METHODS

Isolation of alveolar macrophages. Alveolar macrophages were obtained from specific pathogen-free male Sprague-Dawley rats (225–300 g; Hilltop Laboratories, Scottsdale, PA). The animals were anesthetized with pentobarbital sodium (150 mg/kg body wt) and exsanguinated by cutting the abdominal aorta. The trachea, heart, and lungs were then removed from the animals intact. Alveolar macrophages were obtained via bronchoalveolar lavage according to the method of Myrvik et al. (30). The lungs from each animal were lavaged eight times with 5 ml phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.4 mM Na₂HPO₄, and 1.9 mM NaH₂PO₄, pH 7.4)/g lung weight. The cells were separated from the lavage fluid by centrifugation at 300 *g* for 5 min and then washed three times by alternate centrifugation and resuspension in phosphate-buffered medium. After the washing procedure, the cells were resuspended in the medium used for cell culture (culture medium). The culture medium consisted of Eagle's minimal essential medium (MEM; Bio-Whittaker, Walkersville, MD) supplemented with 1 mM glutamine (GIBCO, Life Technologies, Grand Island, NY), 10 mM HEPES (Sigma, St. Louis, MO), 100 U/ml of penicillin-streptomycin (GIBCO), 100 µg/ml of kanamycin (GIBCO), and 10% (vol/vol) heat-inactivated fetal bovine serum (Bio-Whittaker), pH 7.4. This medium was made in endotoxin-free water (BioWhittaker) and filtered through a 0.2-µm Nalgene bottle top filter (Sybron, Rochester, NY). The number of cells in the suspension was determined by using an electronic cell counter (model Z_B, Coulter Electronics, Hialeah, FL).

Isolation of pulmonary surfactant. Pulmonary surfactant was obtained from a separate set of rats. The trachea, heart, and lungs were removed intact from anesthetized animals as described in *Isolation of alveolar macrophages*. A concentrated form of alveolar lavage material was obtained by bronchoalveolar lavage of the right lung with 5 ml of phosphate-buffered medium followed by lavage of the left lung with the same 5 ml of fluid. Alveolar macrophages were removed from the lavage fluid by centrifugation at 300 *g* for 5 min and then washed three times by alternate centrifugation and resuspension in phosphate-buffered medium. All of these washings were spun at 15,000 *g* for 10 min. The pellet derived from these washings was added back to the remaining cell-free lavage materials because a significant amount of surfactant PLs is recovered in this pellet (28).

Purified lung surfactant was then obtained according to the method of King and Clements (19). Briefly, the cell-free lavage material was spun at 100,000 *g* for 2 h. The resultant pellet was resuspended in phosphate-buffered medium and applied to a linear sodium bromide density gradient (density range 1.028–1.100 g/ml). The gradient was then spun at 81,500 *g* for 15 h in a SW 27 swinging-bucket rotor (Beckman Instruments, Fullerton, CA). The band containing the surfactant (density 1.050 ± 0.003 g/ml) was removed and spun at 66,000 *g* for 1 h. This pellet was washed two times in phosphate-buffered medium and then resuspended in culture medium for use as the surfactant preparation in the experiments. In a separate set of experiments, we determined from osmolality measurements that most of the sodium bromide was removed from the lung surfactant during the wash procedure. PLs were measured as the phosphorus present in the lipid extracts of surfactant (2), and PL content, in milligrams, was obtained by multiplying the lipid phosphorus values by 25 (32).

Isolation of SPs. Three different SPs, SP-A, SP-B, and SP-C, were investigated in this study. SP-A was a gift from Dr. Jo Rae Wright (Duke University, Durham, NC) and was purified from bronchoalveolar lavage material from human alveolar proteinosis patients. Because SP-A is a hydrophilic protein, it was dissolved in culture medium for delivery to the cells. SP-B and SP-C were obtained according to a method previously published (42). The proteins were purified from lipid extracts of bovine lung lavage by chromatography on C-8 silica and Sephadex L-60. Purity of the proteins was determined with SDS-PAGE (visualized by silver stain), amino acid analysis, and compositional analysis (42). The SP-B and SP-C concentrations were determined with the bicinchoninic acid total protein assay (Pierce, Rockford, IL), with 0.1% SDS in all samples and BSA as the standard. Because SP-B and SP-C are hydrophobic proteins, they were incorporated into lipid vesicles for delivery to the cells. The vesicles were composed of some of the lipid components of surfactant, i.e., dipalmitoylphosphatidylcholine (DPPC), L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl (PC), L-α-phosphatidyl-DL-glycerol (PG), and cholesterol. The molar ratio was 10:5:2:3 (DPPC-PC-PG-cholesterol). All lipids were obtained from Sigma. The vesicles were prepared as described previously (20). Briefly, lipids and proteins (SP-B or SP-C) were dissolved in ethanol. Liposomes were formed by injecting the dissolved materials into culture medium warmed to 48°C. Then the dispersion was sonicated to form vesicles, and the vesicles were added to the incubation mixtures.

Measurement of ·NO production. Alveolar macrophages suspended in culture medium were placed into wells of 24-well tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ). One milliliter of culture medium containing 2 × 10⁶ cells was placed in each well. The cells were incubated for 22 h at 37°C in an incubator with a humidified atmosphere (relative humidity 90%) of 95% air-5% CO₂. After the incubation period, the supernatants were removed from each well and spun at 13,000 *g* for 30 s in microfuge tubes to be certain that all cells were removed. The resultant supernatants were saved for analysis. The amount of ·NO in the supernatants was measured as the stable oxidation products of ·NO, nitrate and nitrite. All samples were first incubated with *Escherichia coli* reductase to convert the nitrate to nitrite. ·NO production was then measured by using the Greiss reaction (12). The amount of nitrate and nitrite in the samples was calculated from a standard curve that was constructed from sodium nitrite standards. Conversion of nitrate to nitrite was checked in each assay with sodium nitrate standards.

The effects of LPS, pulmonary surfactant, and different NOS inhibitors on ·NO production by alveolar macrophages were determined. LPS (from *E. coli* 026:B6; Difco Laboratories, Detroit, MI) was added to some wells of the tissue culture plate so that the final concentration was 10 µg/ml, a concentration that produces maximal ·NO formation. Pulmonary surfactant, which had been resuspended in tissue culture medium, was added to some wells so that the final concentration was 25–200 µg PL/ml. Three different NOS inhibitors, N^G-monomethyl-L-arginine acetate (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME), and aminoguanidine hemisulfate, were included in some wells at a final concentration of 1 mM. All inhibitors were obtained from RBI (Natick, MA). In almost all experiments, these substances were added to the wells before the beginning of the incubation period. However, in some experiments, some of these substances were added at different times during the incubation period.

In other experiments, alveolar macrophages were incubated with some of the lipid and protein components of

pulmonary surfactant or with Survanta, a surfactant preparation used in replacement therapy, in an attempt to determine which component(s) is responsible for the surfactant effects. The effects of some of the lipid components of surfactant, DPPC-PC-PG-cholesterol in a molar ratio of 10:5:2:3, on LPS-induced $\cdot$ NO production were determined. The lipids were prepared as vesicles as described in *Isolation of SPs*. The final amount used was 200 μ g PL/ml. In some experiments, only DPPC, the major component of surfactant, was included in the incubation medium. DPPC was prepared as vesicles at a final concentration of 100 μ g/ml because it accounts for ~50% of the PLs in surfactant. In some experiments, Survanta (Ross Laboratories, Columbus, OH) was added to the incubation mixture at a final concentration of 200 μ g PL/ml. The effects of three SPs, SP-A, SP-B, and SP-C, on $\cdot$ NO production by alveolar macrophages were also determined. The proteins were delivered to the cells as described in *Isolation of SPs*. None of the substances used in this study had any effect on the assay for nitrate and nitrite. Also, by doing cell protein measurements and microscopic analysis of the supernatants, we determined that none of the substances affected the ability of the cells to adhere to the culture plates.

Detection of iNOS mRNA. iNOS mRNA levels were determined in untreated alveolar macrophages and in cells exposed to LPS alone or to LPS plus lung surfactant. The mRNA levels were measured by isolating total cellular RNA and then using Northern blot analysis. The cells were incubated for 22 h in culture medium alone, in medium with LPS, or in medium with LPS plus lung surfactant as described in *Measurement of $\cdot$ NO production*. After the incubation period, total cellular RNA was isolated with a guanidinium thiocyanate-based extraction procedure (8) and quantified spectrophotometrically at 260 nm. The relative purities of all RNA samples, as determined by the ratio of absorbance at 260 nm to that at 280 nm (~1.9), were not different. The RNA was then size fractionated on a 1.5% agarose gel containing 2 M formaldehyde and blotted onto a Duralose membrane (Stratagene, La Jolla, CA) with capillary-mediated bulk flow transfer. The amount of total RNA analyzed in each sample was 5–10 μ g. iNOS mRNA was indexed by Northern blot analysis with a 32 P-labeled cDNA hybridization probe derived from a plasmid containing a 4,100-bp cDNA fragment for murine macrophage iNOS (23). The probe was obtained from Drs. C. Lowenstein and S. H. Snyder (Johns Hopkins University, Baltimore, MD). The cDNA fragment for iNOS was amplified by PCR to produce a double-stranded cDNA template (GeneAmp DNA Amplification Reagent Kit, Perkin-Elmer Cetus, Norwalk, CT) with 20-bp synthetic DNA oligonucleotide primers. The sense primer sequence was 5'-ACTTCCTGGACATTACGACC-3', and the antisense primer sequence was 5'-CTGCTCCTCGCTCAAGTTCA-3'. A single-stranded DNA hybridization probe was generated by PCR on the double-stranded cDNA template with the antisense primer and 32 P-labeled dCTP (ICN Biochemicals, Costa Mesa, CA). The hybridization probe was purified on a G-25 Quick Spin column (Boehringer Mannheim, Indianapolis, IN). Northern blot hybridization was performed with QuickHyb hybridization buffer (Stratagene) according to the manufacturer's instructions. The blot was then boiled in RNase-free water for 5 min to remove hybridized probe, and the amount of 28S rRNA on the blot was determined by using the hybridization protocol of Barbu and Dautry (1).

To estimate the amount of iNOS mRNA in the alveolar macrophages, the blots were subjected to image analysis. Images were obtained with a video camera (model CCD72, Dage-MTI, Michigan City, IN) and projected on a video monitor. The images were then taken from the monitor with a

frame grabber card (model MVP AT, Matrox Electronic Systems, Dorval, PQ). The software used for these measurements was Optimas, which was obtained from Bioscan (Edmonds, WA). With this software, the computer was allowed to define the areas of interest and to compute the total (integrated) gray value, which is a measure of both the intensity and the area of color. Individual iNOS mRNA signal levels were then divided by the corresponding 28S rRNA sample signal level to normalize the values for the amount of RNA loaded. For each experiment, the iNOS mRNA level for cells exposed to LPS alone was taken to be 100%. The iNOS mRNA level for cells exposed to both LPS and surfactant is expressed as a percentage of that for cells exposed to LPS alone. In all cases, the range of darkness for the scanned images was between 30 and 75% of the full scale (100% = darkest). Therefore, image analysis was performed in the midrange of the darkness scale.

Detection of iNOS protein. To determine whether iNOS protein could be detected in alveolar macrophages exposed to LPS alone, to LPS plus lung surfactant, or to LPS plus SP-B, Western blot analysis was used. Alveolar macrophages were incubated for 22 h in culture medium with LPS, LPS plus pulmonary surfactant, or LPS plus SP-B as described in *Measurement of $\cdot$ NO production*. After the incubation period, the supernatants were removed and saved for analysis of nitrate and nitrite. The cells were removed from the culture plates and used for Western blot analysis. SDS-PAGE was performed on 100- μ g aliquots of cell protein with 7.5% (wt/vol) polyacrylamide gels. Proteins were transferred to nitrocellulose paper with an electrophoretic transfer unit (Hoefer Scientific Instruments, San Francisco, CA). The blots were then blocked for 1 h at room temperature in a medium (blocking buffer) containing 50 mM Tris-HCl, 150 mM NaCl, 2% (vol/vol) BSA, and 0.1% (vol/vol) Tween 20, pH 7.4. These blots were then incubated for an additional hour at room temperature in blocking buffer containing anti-iNOS antibody. The primary antibody used was mouse macrophage (IgG2a) monoclonal anti-iNOS (Transduction Laboratories, Lexington, KY). The antibody was diluted 1:500 in blocking buffer. After incubation with the primary antibody, the blots were washed six times (5 min/wash) at room temperature with medium containing 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 (Tris-buffered saline-Tween 20; pH 7.4). Then the blots were incubated for 1 h at room temperature in blocking buffer containing the secondary antibody, anti-mouse IgG coupled to horseradish peroxidase (Amersham Life Sciences, Cleveland, OH). After incubation with the secondary antibody, the blots were washed six times (5 min/wash) at room temperature with Tris-buffered saline-Tween 20. Protein bands detected by the antibody were visualized by enhanced chemiluminescence (Amersham Life Sciences). The standard that was carried through the entire procedure was macrophage lysate prepared from RAW 264.7 cells that had been stimulated with IFN- γ and LPS (Transduction Laboratories). To estimate the relative amounts of iNOS protein in the alveolar macrophages, the Western blots were subjected to image analysis as described in *Detection of iNOS mRNA*. For each experiment, the protein level for the cells exposed to LPS alone was taken to be 100%. The iNOS protein level for cells exposed to LPS plus surfactant or to LPS plus SP-B is expressed as a percentage of that for cells exposed to LPS alone.

Measurement of iNOS activity. The effects of pulmonary surfactant on LPS-induced iNOS activity in alveolar macrophages were studied by measuring the conversion of L-arginine to L-citrulline (6). Alveolar macrophages were incubated with LPS for 22 h in culture medium as described

in *Measurement of $\cdot$ NO production*. After the incubation period, the cells were removed from the culture plates and suspended in ice-cold medium containing 50 mM Tris·HCl, 12 mM mercaptoethanol, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 2 μ M leupeptin, 1 μ M pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, pH 7.0. A crude preparation of cellular cytosol and microsomes was made by sonicating the suspension twice for 10 s each time and then spinning at 13,000 *g* for 1 min in an Eppendorf microfuge. The supernatants were removed and frozen at -80°C until used for assay.

To measure conversion of L-arginine to L-citrulline, 50 μ l of the cell cytosolic and microsomal preparation (containing 50 μ g of protein) were added to 50 μ l of a medium containing 67 mM Tris·HCl, 3.3 mM CaCl_2 , 2.0 mM NADPH, 4 μ M flavin mononucleotide, 4 μ M FAD, 20 U of calmodulin, and 20 μ M tetrahydrobiopterin, pH 7.2. Some samples contained either pulmonary surfactant (25 μ g PL/ml) or aminoguanidine (1 mM). The reaction was initiated by adding 0.15 μ Ci of L-[2,3,4,5- ^3H]arginine monohydrochloride (specific activity 61 Ci/mmol; Amersham Life Sciences). Incubations were carried out at 37°C for 10 min. The concentration of protein and time of incubation used were within linear ranges. After the incubation period, 400 μ l of sodium HEPES buffer (30 mM sodium HEPES and 3 mM EDTA) were added to the incubation mixture that was then placed on ice. Then 400 μ l of a 50% (wt/vol) slurry of Dowex (Dowex AG 50W-8X, sodium form, Bio-Rad Laboratories, Hercules, CA) were added to the samples, and the samples were vortexed for 20 s. The Dowex was removed by spinning the samples at 13,000 *g* for 2 min in the microfuge. An aliquot (0.5 ml) of the supernatant was then added to 4 ml of scintillation fluid (EnviroSafe, Anoroc Scientific, Hackensack, NJ), and the samples were counted in a liquid scintillation counter. The results are expressed as picomoles of L-citrulline produced per milligram of protein.

Generation of $\cdot$ NO by spermine/NO complex. $\cdot$ NO was produced by a $\cdot$ NO generator in a cell-free system to determine whether pulmonary surfactant can act as an antioxidant or radical scavenger and reduce the amount of $\cdot$ NO formed. Spermine/NO complex (RBI) was incubated for 1 h in culture medium maintained at 37°C in 95% air-5% CO_2 (relative humidity 90%). The final concentration of spermine/NO complex was 0.2 mM because this concentration produces approximately the same amount of $\cdot$ NO as that formed by 2×10^6 LPS-stimulated alveolar macrophages, the cell number used in 1 ml of all our incubation mixtures. Incubations were carried out with spermine/NO complex alone or with spermine/NO complex plus lung surfactant (200 μ g PL/ml). After the incubation period, the samples were removed, and nitrate and nitrite was measured as described in *Measurement of $\cdot$ NO production*.

Statistical analyses. All comparisons of significance were made by comparing each treatment with the control value (100%) with a one-sample Student's *t*-test. $P < 0.05$ was taken as the limit to indicate significance.

RESULTS

NOS inhibitor effects on LPS-induced $\cdot$ NO production. The effects of three different NOS inhibitors on LPS-induced $\cdot$ NO formation by alveolar macrophages were determined, and the results are shown in Fig. 1. Incubation of the cells with L-NAME, L-NMMA, or aminoguanidine led to inhibition of LPS-induced $\cdot$ NO production by 71, 93, or 83%, respectively. These results are much different from the inhibitor effects on the $\cdot$ NO produced by a constitutive NOS (cNOS) in rat

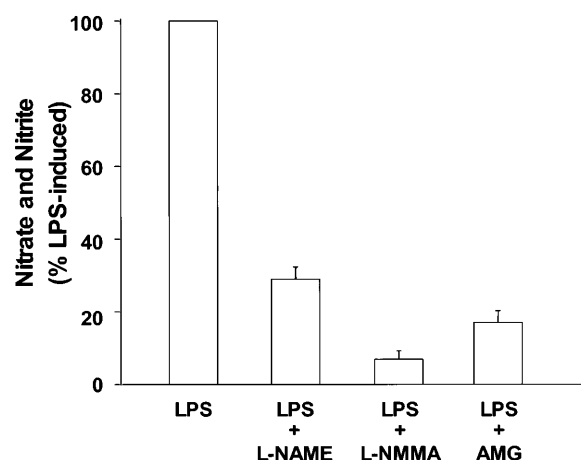


Fig. 1. Effects of nitric oxide ($\cdot$ NO) synthase inhibitors on lipopolysaccharide (LPS)-induced $\cdot$ NO production by alveolar macrophages. Cells (2×10^6 /ml) were incubated for 22 h in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO_2 (relative humidity 90%). Alveolar macrophages were incubated with LPS (10 μ g/ml) alone or with LPS + N^G -monomethyl-L-arginine (L-NMMA), N^G -nitro-L-arginine methyl ester (L-NAME), or aminoguanidine (AMG). Final concentration of all inhibitors was 1 mM. After incubation period, nitrate and nitrite in supernatants were measured as described in METHODS. Values are means $\pm$ SE for 6 experiments and are expressed as percentage of $\cdot$ NO production by cells exposed to LPS alone (%LPS induced). Nitrate and nitrite production by cells exposed to LPS alone was 108 ± 4 nmol/ 10^6 cells.

alveolar macrophages, where only L-NAME inhibited production by only 32% (27). L-NAME and L-NMMA are inhibitors of both iNOS and cNOS (35), whereas aminoguanidine is specific for iNOS (13). Previous results by Miles et al. seem to confirm the specificity of aminoguanidine for iNOS in lung cells in that it did not inhibit $\cdot$ NO formed by cNOS in either alveolar macrophages (27) or alveolar type II cells (26). Thus all of these results appear to confirm that the $\cdot$ NO produced by alveolar macrophages in response to LPS is due to iNOS.

Pulmonary surfactant effects on LPS-induced $\cdot$ NO production. The effects of pulmonary surfactant on $\cdot$ NO production by alveolar macrophages were investigated. When alveolar macrophages were incubated in culture medium alone for 22 h, very little nitrate plus nitrite was formed, i.e., 0.36 ± 0.12 (SE) nmol/ 10^6 cells ($n = 6$ experiments). If the cells were incubated with lung surfactant (200 μ g PL/ml), more nitrate plus nitrite was produced, i.e., 3.9 ± 1.9 nmol/ 10^6 cells. However, when the cells were incubated with LPS (10 μ g/ml), there was a great increase in the nitrate plus nitrite formed, i.e., 108 ± 4 nmol/ 10^6 cells. Incubation of alveolar macrophages with LPS in the presence of lung surfactant led to a concentration-dependent inhibition of LPS-induced $\cdot$ NO formation. These results are shown in Fig. 2. $\cdot$ NO production was inhibited by $\sim 50\%$ at surfactant levels of ~ 100 μ g PL/ml and by $\sim 75\%$ at the highest surfactant level used, 200 μ g PL/ml. These results demonstrate that pulmonary surfactant inhibits LPS-induced $\cdot$ NO production by alveolar macrophages in a concentration-dependent manner. Many of the remaining experiments described in this paper

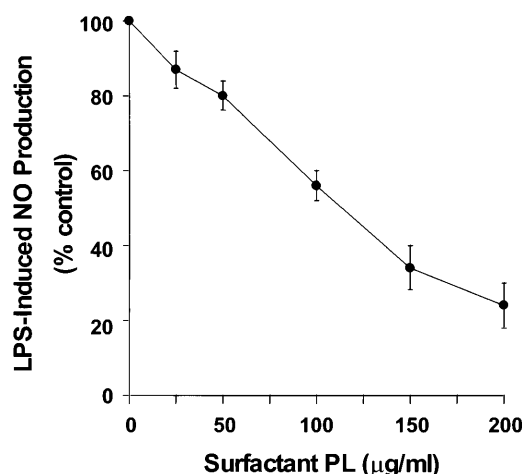


Fig. 2. Effects of different amounts of pulmonary surfactant on LPS-induced $\cdot$ NO production by alveolar macrophages. Cells (2×10^6 /ml) were incubated for 22 h in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Some cells were exposed to LPS (10 µg/ml) alone, and other cells were exposed to LPS (10 µg/ml) and varying amounts of lung surfactant. Amounts of lung surfactant are expressed as µg phospholipid (PL)/ml. After incubation period, nitrate and nitrite in supernatants were measured as described in METHODS. Values are means $\pm$ SE for 6 experiments and are expressed as percentage of that produced in presence of LPS alone (%control). Nitrate and nitrite production by cells exposed to LPS alone was 108 ± 4 nmol/10⁶ cells.

were designed to study the mechanism by which surfactant inhibits $\cdot$ NO formation. In these experiments, the concentration of surfactant used was 200 µg PL/ml.

Experiments were performed to determine whether surfactant inhibits LPS-induced $\cdot$ NO production by interfering with the ability of LPS to initiate the events involved in the induction of iNOS, e.g., the binding of LPS to the cell membrane. In these experiments, we compared the ability of surfactant to inhibit $\cdot$ NO formation when the cells were incubated for 22 h with LPS and surfactant together with its ability to inhibit $\cdot$ NO production when the cells were exposed to surfactant after stimulation with LPS. In the latter situation, the cells were incubated with LPS for 2 h. The LPS was then washed from the cells, either medium alone or surfactant was added, and the incubations were carried out for an additional 20 h. The results are shown in Table 1. Although there is less $\cdot$ NO produced by cells exposed to LPS for only 2 h, the effects of surfactant are the same in both cases; i.e., surfactant inhibits LPS-induced $\cdot$ NO production by 70–76%. Therefore, these results show that surfactant does not inhibit LPS-induced $\cdot$ NO production by interfering with the initial events in the process, such as LPS binding to the membrane.

Pulmonary surfactant effects on iNOS mRNA. Northern blot analysis was used to determine whether lung surfactant interferes with LPS-induced formation of iNOS mRNA. A representative Northern blot is shown in Fig. 3. This result demonstrates that there was no iNOS message in cells incubated in culture medium alone (Fig. 3, lane 1). Exposure of the cells to LPS led to

Table 1. Effects of altering incubation time with LPS and pulmonary surfactant on $\cdot$ NO production by alveolar macrophages

Treatment	Nitrate + Nitrite, nmol/10 ⁶ cells
LPS (22 h)	100 $\pm$ 6
LPS + lung surfactant (22 h)	24 $\pm$ 6*
LPS (2 h); wash; medium alone (20 h)	68 $\pm$ 4
LPS (2 h); wash; lung surfactant (20 h)	20 $\pm$ 7*

Values are means $\pm$ SE of 6 experiments; nos. in parentheses, incubation times. LPS, lipopolysaccharide; $\cdot$ NO, nitric oxide. Alveolar macrophages (2×10^6 /ml) were incubated in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Cells were incubated with LPS alone, with LPS+lung surfactant, or with LPS, then washed free of LPS and incubated with only culture medium or with medium containing lung surfactant. Amount of LPS and lung surfactant used in all cases was 10 µg/ml and 200 µg phospholipid (PL)/ml, respectively. After final incubation period, nitrate and nitrite in supernatants were measured as described in METHODS. *Significantly different from value for $\cdot$ NO production in absence of surfactant, $P < 0.05$.

a substantial level of iNOS mRNA (Fig. 3, lane 2), and the addition of lung surfactant had no effect on LPS-induced iNOS mRNA (Fig. 3, lane 3). All blots were subjected to image analysis to estimate the relative amounts of iNOS mRNA in cells exposed to LPS alone and in cells exposed to LPS plus surfactant. The results are shown in Table 2. Incubation of the alveolar macrophages with LPS plus surfactant led to iNOS mRNA levels that were no different from the mRNA levels in the presence of LPS alone. In addition, we also obtained similar results by performing PCR on serial dilutions of reverse-transcribed DNA. Thus these results show that

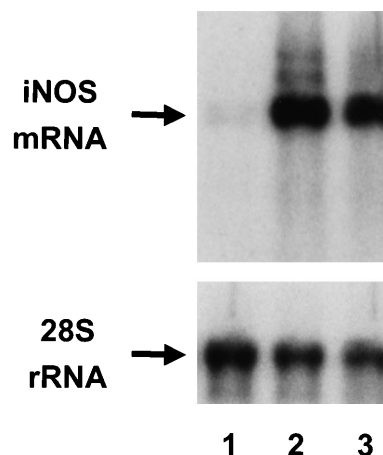


Fig. 3. Northern blot analysis of inducible $\cdot$ NO synthase (iNOS) mRNA and 28S rRNA obtained from untreated alveolar macrophages (lane 1) and cells treated with LPS alone (lane 2) or LPS+pulmonary surfactant (lane 3). Cells (2×10^6 /ml) were incubated for 22 h in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Alveolar macrophages were incubated alone (control), with LPS (10 µg/ml), or with LPS+lung surfactant (200 µg PL/ml). After incubation period, cells were removed from culture plates, and total cellular RNA was isolated and analyzed for iNOS mRNA expression by Northern blot analysis as described in METHODS. Blot is representative of results obtained from 7 different alveolar macrophage preparations.

Table 2. Effects of pulmonary surfactant on LPS-induced iNOS mRNA levels in alveolar macrophages

Treatment	iNOS mRNA, %LPS induced
LPS	100
LPS + lung surfactant	92 ± 6

Values are means ± SE of 7 experiments. iNOS, inducible NO synthase. Alveolar macrophages (2×10^6 /ml) were incubated in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Cells were incubated for 22 h in presence of LPS (10 µg/ml) or LPS+lung surfactant (200 µg PL/ml). After incubation period, cells were removed from culture plates. Northern blot analysis was performed on aliquots of total cellular RNA, and image analysis was used to estimate amount of iNOS mRNA as described in METHODS. Results obtained in presence of LPS+lung surfactant are expressed as a percentage of those obtained in presence of LPS alone.

lung surfactant had no effect on the amount of LPS-induced iNOS mRNA in alveolar macrophages.

Pulmonary surfactant effects on iNOS protein. Experiments were performed in an attempt to determine whether exposure of alveolar macrophages to pulmonary surfactant led to changes in the amount of iNOS protein produced in response to LPS. Western blot analysis was used. The results, which are shown in Fig. 4, demonstrate the presence of an LPS-induced alveolar macrophage protein that reacted with a monoclonal

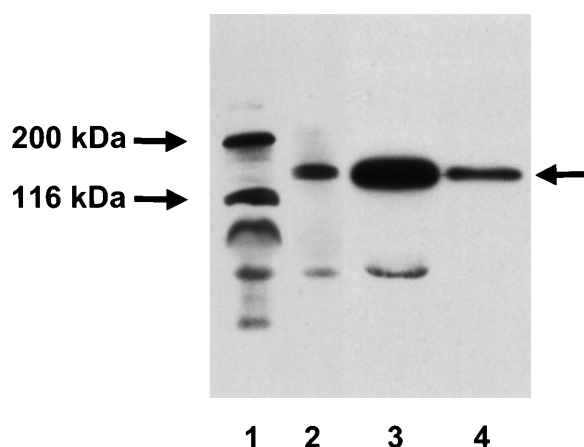


Fig. 4. Western blot analysis of alveolar macrophage proteins with anti-iNOS antibody. Cells (2×10^6 /ml) were incubated for 22 h in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Alveolar macrophages were incubated with LPS (10 µg/ml) alone or with LPS+lung surfactant (200 µg PL/ml). After incubation period, cells were removed from culture plates, and membranes were disrupted as described in METHODS. SDS-PAGE was used to fractionate 100-µg aliquots of cell protein. Proteins were then transferred to a nitrocellulose membrane and immunodetected with a monoclonal anti-iNOS antibody. Lane 1, molecular-mass markers (nos. at left); lane 2, standard preparation of iNOS from RAW 264.7 cells that had been stimulated with LPS and interferon-γ was used as a positive control and was carried through entire procedure; lane 3, proteins from cells exposed to LPS alone; lane 4, proteins from cells exposed to LPS+lung surfactant. Arrow at right location of iNOS at a molecular mass of ~120 kDa. Blot is representative of results obtained from 6 different alveolar macrophage preparations.

anti-iNOS antibody (lane 3). The molecular mass of this protein (~120 kDa) corresponds to that of an iNOS standard that was obtained from macrophage lysate prepared from RAW 264.7 cells that had been stimulated with IFN-γ and LPS (Fig. 4, lane 2). No iNOS protein was detected in cells incubated without LPS or with lung surfactant alone (data not shown). However, when the cells were incubated with LPS plus surfactant, there was less iNOS protein present (Fig. 4, lane 4) than when the cells were incubated with LPS alone (Fig. 4, lane 3). The blots were subjected to image analysis to estimate the relative amounts of iNOS protein present (Table 3). The results show that incubation of alveolar macrophages with lung surfactant led to 80–90% decreases in LPS-induced iNOS protein levels and NO production. Therefore, the surfactant-induced inhibition of LPS-stimulated NO formation appears to be related to a reduction in the amount of iNOS protein.

Pulmonary surfactant effects on iNOS activity. To determine whether lung surfactant can inhibit iNOS activity, the conversion of L-arginine to L-citrulline was measured in a crude preparation of cytosol and microsomes obtained from alveolar macrophages that had been incubated with LPS for 22 h. The results are shown in Table 4. When the preparation was incubated alone (with no surfactant), there were ~3 pmol L-citrulline produced/mg protein in 10 min. There was no change in iNOS activity when lung surfactant was included in the incubation mixture. It should be noted that we used the same ratio of lung surfactant to cellular protein in these experiments as was used in the incubation of intact alveolar macrophages with surfactant in the experiments described in *Measurement of iNOS activity*. The iNOS inhibitor aminoguanidine was used as a positive control. Aminoguanidine (1 mM) led to a 98% reduction in iNOS activity. These results demonstrate that lung surfactant does not affect iNOS activity in LPS-induced alveolar macrophages, at least in vitro.

Table 3. Effects of pulmonary surfactant on LPS-induced NO production and iNOS protein levels in alveolar macrophages

Treatment	Nitrate + Nitrite, %LPS induced	iNOS Protein, %LPS induced
LPS	100	100
LPS + lung surfactant	13 ± 4*	20 ± 8*

Values are means ± SE of 6 experiments. Alveolar macrophages (2×10^6 /ml) were incubated in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Cells were incubated for 22 h in presence of LPS (10 µg/ml) or LPS+lung surfactant (200 µg PL/ml). After incubation period, nitrate and nitrite in supernatants were measured as described in METHODS. Cells were removed from culture plates, Western blot analysis was performed on aliquots of cellular protein, and image analysis was performed on blots as described in METHODS. Results obtained in presence of LPS+lung surfactant are expressed as a percentage of those in presence of LPS alone. Nitrate + nitrite level in cells exposed to LPS alone was 97 ± 6 nmol/10⁶ cells. *Significantly different from treatment with LPS alone, $P < 0.05$.

Table 4. *Effects of pulmonary surfactant on iNOS activity in LPS-stimulated alveolar macrophages*

Treatment	Formation of L-Citrulline, %control
Control	100
Lung surfactant	109 ± 10
Aminoguanidine	2 ± 1*

Values are means ± SE of 6 experiments. Alveolar macrophages (2×10^6 /ml) were incubated in culture medium with LPS (10 µg/ml) for 22 h as described in METHODS. After incubation period, cells were removed from culture plates, and a crude preparation of cell cytosol and microsomes was made as described in METHODS. iNOS activity was determined by measuring conversion of L-arginine to L-citrulline. Fifty microliters of cell cytosol and microsomal preparation (containing 50 µg of protein) were added to 50 µl of a medium containing 67 mM Tris·HCl, 3.3 mM CaCl₂, 2.0 mM NADPH, 4 µM flavin mononucleotide, 4 µM FAD, 20 U of calmodulin, and 20 µM tetrahydrobiopterin. Some samples contained lung surfactant (25 µg of PL) or aminoguanidine (1 mM). Reactions were initiated by adding 0.15 µCi of L-[³H]arginine. Incubations were carried out for 10 min. Conversion of L-arginine to L-citrulline was determined as described in METHODS. Formation of L-citrulline in control experiments was 3.0 ± 0.4 pmol/mg protein. *Significantly different from control value, $P < 0.05$.

Pulmonary surfactant effects on ·NO generation by spermine/NO complex. ·NO was produced by an ·NO generator in a cell-free system to determine whether pulmonary surfactant can reduce the amount of ·NO formed by acting as an antioxidant or radical scavenger. Spermine/NO complex was used to generate ·NO. The concentration of the generator was adjusted so that the ·NO produced was approximately the same as that formed by the number of LPS-stimulated cells placed in one well of the culture plates, i.e., as in all other experiments in which ·NO production was measured. The amount of lung surfactant used in these experiments was approximately the same as the greatest amount used to inhibit ·NO formation by the intact cells in one well of the culture plates. The results are shown in Table 5. ·NO production by spermine/NO complex was not affected by lung surfactant. Similar results were obtained with another ·NO generator, S-nitroso-N-acetylpenicillamine (data not presented). These results suggest that surfactant does not inhibit ·NO production from LPS-stimulated alveolar macrophages by acting as an antioxidant or radical scavenger.

Table 5. *Effects of pulmonary surfactant on ·NO levels produced by ·NO generator spermine/NO complex*

Treatment	Nitrate + Nitrite, %spermine/NO complex
Spermine/NO complex	100
Spermine/NO complex + lung surfactant	102 ± 2

Values are means ± SE of 6 experiments. Spermine/NO complex (0.2 mM) was incubated in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Incubations were carried out for 1 h in presence of spermine/NO complex alone or with spermine/NO complex+lung surfactant (200 µg PL/ml). After incubation period, nitrate and nitrite in samples were measured as described in METHODS. Nitrate + nitrite production in presence of spermine/NO complex alone was 103 ± 5 nmol/0.5 ml.

Effects of pulmonary surfactant components on LPS-induced ·NO production. Experiments were performed in an attempt to determine which component(s) of lung surfactant is responsible for the inhibition of LPS-induced ·NO production by alveolar macrophages. The results are shown in Table 6. The effects of whole lung surfactant, which produces ~75% inhibition at a concentration of 200 µg/ml, have been described (Fig. 2) and are shown here for the sake of comparison. Incubation of the cells with the major component of surfactant, DPPC, had no effect on LPS-induced ·NO formation. The concentration of DPPC used was 100 µg/ml because it accounts for ~50% of the PLs in surfactant. We also determined that incubating the cells with a mixture of some surfactant lipids (mixed lipids; DPPC, unsaturated PC, PG, and cholesterol in a molar ratio of 10:5:2:3; final concentration 200 µg PL/ml) had no effect. The major hydrophilic surfactant-associated protein, SP-A, also had no effect on LPS-induced ·NO production at concentrations up to 40 µg/ml. In addition, inclusion of both mixed lipids and SP-A in the incubation medium had no effect. On the other hand, Survanta, a commercial surfactant preparation used for replacement therapy, inhibited LPS-induced ·NO formation by ~40% at a concentration of 200 µg PL/ml, i.e., the same PL concentration as was used for whole lung surfactant.

Survanta contains some of the same lipid components that are present in natural surfactant, and it also contains the hydrophobic SPs SP-B and SP-C. There is no SP-A in Survanta. Thus it may be that the hydrophobic proteins are responsible for the surfactant-induced inhibition of ·NO formation. Therefore, experiments were performed to determine the effects of SP-B and SP-C on LPS-induced ·NO production by alveolar mac-

Table 6. *Effects of pulmonary surfactant components and Survanta on LPS-induced ·NO production by alveolar macrophages*

Treatment	Nitrate + Nitrite, %LPS induced
LPS	100
LPS + lung surfactant	25 ± 5*
LPS + DPPC	99 ± 3
LPS + mixed lipids	99 ± 2
LPS + SP-A	101 ± 3
LPS + mixed lipids + SP-A	98 ± 3
LPS + Survanta	61 ± 4*

Values are means ± SE of 6 experiments. Alveolar macrophages (2×10^6 /ml) were incubated in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO₂ (relative humidity 90%). Cells were incubated for 22 h in presence of LPS (10 µg/ml) alone, LPS+lung surfactant (200 µg PL/ml), LPS+dipalmitoylphosphatidylcholine (DPPC; 100 µg/ml), LPS+mixed lipids (200 µg PL/ml), LPS+surfactant protein (SP) A (40 µg/ml), LPS+mixed lipids+SP-A, or LPS+Survanta (200 µg PL/ml). Mixed lipids consisted of DPPC, unsaturated phosphatidylcholine, phosphatidylglycerol, and cholesterol in a molar ratio of 10:5:2:3. After incubation period, nitrate and nitrite in supernatants were measured as described in METHODS. Nitrate + nitrite level in cells exposed to LPS alone was 102 ± 6 nmol/10⁶ cells. *Significantly different from treatment with LPS alone, $P < 0.05$.

rophages. For these experiments, different amounts of the proteins were incorporated into mixed lipid vesicles (200 μg PL/ml) for delivery to the cells. Incubation of alveolar macrophages with SP-C at concentrations up to 20 $\mu\text{g}/\text{ml}$ had no effect on LPS-induced $\cdot\text{NO}$ production; i.e., $\cdot\text{NO}$ formation in the presence of LPS plus SP-C was $99 \pm 4\%$ of that in the presence of LPS alone ($n = 6$ experiments). On the other hand, LPS-induced $\cdot\text{NO}$ formation is inhibited by SP-B in a concentration-dependent manner (Fig. 5). $\cdot\text{NO}$ production is inhibited by ~ 50 and $\sim 90\%$ at SP-B concentrations of 1–2 and 10 $\mu\text{g}/\text{ml}$, respectively. Therefore, all of these results taken together indicate that the component responsible for surfactant-induced inhibition of LPS-stimulated $\cdot\text{NO}$ production is SP-B.

SP-B effects on iNOS protein. Incubation of alveolar macrophages with lung surfactant led to inhibition of LPS-stimulated $\cdot\text{NO}$ formation and iNOS protein levels. The data presented in *Effects of pulmonary surfactant components on LPS-induced $\cdot\text{NO}$ production* show that SP-B inhibited LPS-induced $\cdot\text{NO}$ formation. Therefore, experiments were performed to determine the effects of SP-B on the amount of LPS-induced iNOS protein in alveolar macrophages. Western blot analysis was used. The results, which are shown in Table 7, demonstrate that incubation of the cells with SP-B (10 $\mu\text{g}/\text{ml}$, an amount that produces $\sim 90\%$ inhibition of LPS-induced $\cdot\text{NO}$ production) led to an $\sim 80\%$ reduction in LPS-induced iNOS protein levels. Thus this result suggests that the surfactant component responsible for

Table 7. Effects of SP-B on LPS-induced iNOS protein levels in alveolar macrophages

Treatment	iNOS Protein, %LPS induced
LPS	100
LPS + SP-B	$22 \pm 9^*$

Values are means $\pm$ SE of 6 experiments. Alveolar macrophages ($2 \times 10^6/\text{ml}$) were incubated in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO_2 (relative humidity 90%). Cells were incubated for 22 h in presence of LPS (10 $\mu\text{g}/\text{ml}$) or LPS+SP-B (10 $\mu\text{g}/\text{ml}$) incorporated into lipid vesicles as described in METHODS. After incubation period, cells were removed from culture plates. Western blot analysis was performed on aliquots of cellular protein, and image analysis was performed on blots as described in METHODS. Results obtained in presence of LPS+SP-B are expressed as a percentage of those obtained in presence of LPS alone. *Significantly different from treatment with LPS alone, $P < 0.05$.

surfactant-induced reduction in LPS-induced iNOS protein is SP-B.

DISCUSSION

The results of our experiments demonstrate that pulmonary surfactant inhibits LPS-induced $\cdot\text{NO}$ production by rat alveolar macrophages in a concentration-dependent manner. At the highest level of surfactant used (200 μg PL/ml), LPS-induced $\cdot\text{NO}$ formation is reduced by 75–85%. The inhibition appears to be due to a surfactant-induced reduction in the amount of enzyme responsible for $\cdot\text{NO}$ production, i.e., iNOS. In the presence of surfactant, iNOS protein levels are reduced by $\sim 80\%$. The component of surfactant that appears to be responsible for inhibiting LPS-induced $\cdot\text{NO}$ formation is SP-B, one of the surfactant-associated hydrophobic proteins. SP-B inhibits $\cdot\text{NO}$ production in a concentration-dependent manner. At the highest SP-B level we used, 10 $\mu\text{g}/\text{ml}$, $\cdot\text{NO}$ formation was reduced by $\sim 90\%$.

In addition to a reduction in iNOS protein levels, there are some other possible ways by which surfactant could inhibit LPS-induced $\cdot\text{NO}$ formation. However, our results seem to rule out these possibilities. Experiments in which alveolar macrophages are stimulated with LPS before the addition of surfactant suggest that surfactant does not interfere with the initial events in the process of iNOS induction by LPS, such as LPS binding to the membrane (Table 1). This is also supported by the fact that iNOS mRNA levels are not reduced in the presence of surfactant (Table 2). We have also obtained evidence that surfactant does not directly affect iNOS activity by showing that it has no effect in vitro on the enzyme obtained from LPS-stimulated cells (Table 4). Finally, surfactant has no effect on the $\cdot\text{NO}$ produced by a cell-free generating system, spermine/ NO complex (Table 5). This result indicates that the surfactant reduction in $\cdot\text{NO}$ formation is probably not due to an antioxidant or radical scavenging effect. Thus all of these data taken together suggest that surfactant inhibits LPS-induced $\cdot\text{NO}$ production by causing a decrease in alveolar macrophage iNOS protein levels.

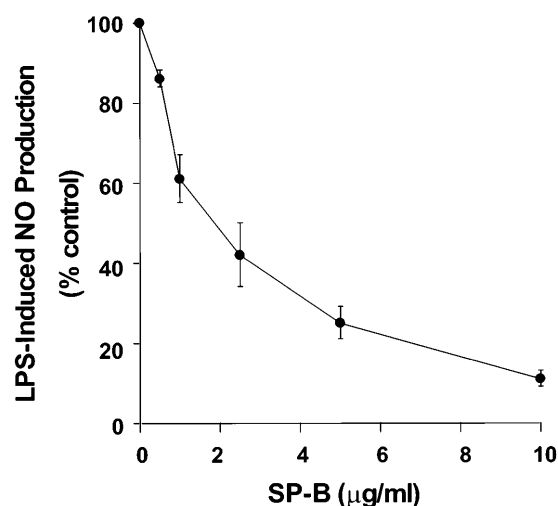


Fig. 5. Effects of different concentrations of surfactant protein (SP) B on LPS-induced $\cdot\text{NO}$ production by alveolar macrophages. Cells ($2 \times 10^6/\text{ml}$) were incubated for 22 h in culture medium (MEM supplemented with glutamine, HEPES, penicillin-streptomycin, kanamycin, and fetal bovine serum) maintained at 37°C in 95% air-5% CO_2 (relative humidity 90%). Some cells were exposed to LPS (10 $\mu\text{g}/\text{ml}$) alone, and other cells were exposed to LPS (10 $\mu\text{g}/\text{ml}$) and varying amounts of SP-B. SP-B was incorporated into lipid vesicles for delivery to cells as described in METHODS. Lipid vesicles alone had no effect on $\cdot\text{NO}$ production. After incubation period, nitrate and nitrite in supernatants were measured as described in METHODS. Values are means $\pm$ SE for 6 experiments and are expressed as percentage of that produced in presence of LPS alone. Nitrate and nitrite production by cells exposed to LPS alone was 98 ± 6 nmol/ 10^6 cells.

Our data suggest that the effects of surfactant on iNOS protein levels may be posttranscriptional and/or posttranslational. We have shown that iNOS mRNA levels are not affected by surfactant. It may be that the message is not translated so that iNOS protein synthesis does not occur. If this is the case, the surfactant effect would be posttranscriptional. Under normal conditions, the iNOS protein level is determined by a balance between its rate of synthesis and its rate of degradation. Thus another possibility is that translation occurs so that iNOS protein is synthesized and that its reduction is due to an increase in the rate of degradation. In this case, the effect would be posttranslational. Of course, it is also possible that the surfactant effect is a combination of posttranscriptional and posttranslational effects. At this time, it is not possible to ascertain which of these effects is responsible for surfactant-induced inhibition of $\cdot\text{NO}$ production.

The surfactant component responsible for the inhibition of LPS-induced $\cdot\text{NO}$ production appears to be SP-B. This protein inhibits $\cdot\text{NO}$ formation in a concentration-dependent manner. At the highest concentration we used, 10 $\mu\text{g/ml}$, $\cdot\text{NO}$ production was inhibited by $\sim 90\%$. Hull et al. (16) measured the concentrations of SP-B and PL in human bronchoalveolar lavage fluid. Their results show that the weight ratio of SP-B to PL is $\sim 1:10$. If this is the case for our surfactant preparations, an SP-B concentration of 10 $\mu\text{g/ml}$ is approximately one-half of that present in the highest surfactant levels used in our incubations (i.e., 200 $\mu\text{g PL/ml}$). These calculations suggest that there is sufficient SP-B in surfactant to account for the inhibition of LPS-induced $\cdot\text{NO}$ formation. Furthermore, we calculated that the SP-B level in natural surfactant is 3- to 10-fold greater than that in Survanta (Miles and Baatz, personal observations), which probably accounts for the greater inhibitory effect of natural surfactant. None of the other surfactant components we studied has any effect on $\cdot\text{NO}$ production. The major lipid components, SP-A, and SP-C had no effect. The other hydrophilic surfactant protein, SP-D, was not examined in the present study.

Almost all previous studies done with regard to SP-B have been concerned with its role in surfactant metabolism and function (see Ref. 15 for a review). For example, it has been shown that this protein is important in the intra-alveolar ordering of surfactant lipids into tubular myelin after their secretion from alveolar type II cells (34, 37). SP-B also plays an important role in making the surfactant readily spreadable and in stabilizing the film at the air-liquid interface (10, 38). Also, this protein stimulates the reuptake of phospholipids by type II cells (9, 36), suggesting that it is involved in surfactant reutilization. As far as we know, the results of our present study with regard to SP-B are unique in that they represent the first reported effect of SP-B that apparently is not directly related to surfactant metabolism or function. It also represents the first reported effect of this protein on alveolar macrophages and suggests that SP-B may play a role in lung defense.

The exact mechanism by which SP-B inhibits LPS-induced $\cdot\text{NO}$ production is not known. We have shown

that there is a reduction in the iNOS protein level and suggested that this effect may be posttranscriptional and/or posttranslational. It may be that SP-B is taken into the cells along with surfactant lipids and then exerts its effects intracellularly. In this regard, Miles et al. (29) have shown that natural surfactant and lipid vesicles are readily taken up to the same extent by alveolar macrophages. Furthermore, Breslin and Weaver (7) studied the uptake of SP-B by alveolar type II cells. Their data suggest that SP-B is internalized by a pathway similar to PLs. These results suggest that the protein may exert its effect inside the cells. Alternatively, SP-B may exert its effects via a cell surface receptor, although an SP-B-specific macrophage cell surface receptor has not been identified as yet.

One result from our study differs from that reported by Blau et al. (5). They found that $\cdot\text{NO}$ production by rat alveolar macrophages is upregulated by SP-A. One source of their SP-A was human alveolar proteinosis patients. In this regard, our SP-A was obtained from a similar source and isolated in a manner similar to that used by Blau et al. However, SP-A had no effect on $\cdot\text{NO}$ formation by alveolar macrophages in our hands. In most of our experiments, we used an SP-A concentration of 20–40 $\mu\text{g/ml}$, whereas Blau et al. found a maximal effect at 4 $\mu\text{g/ml}$. When we did use SP-A concentrations as low as 4 $\mu\text{g/ml}$, there was no effect on cellular $\cdot\text{NO}$ production (data not shown). The reasons for these contrasting effects of SP-A are not known.

The significance of surfactant effects on LPS-induced $\cdot\text{NO}$ formation by alveolar macrophages is not known. However, other investigators have reported that various surfactant preparations or components of surfactant have inhibitory effects on the release of cytokines from alveolar macrophages. For example, surfactant preparations used in replacement therapy, Exosurf and Survanta, suppress the endotoxin-induced production of three cytokines, TNF- α , IL-1 β , and IL-6 (39, 40). The major hydrophilic SP, SP-A, also inhibits the release of TNF- α from LPS-stimulated alveolar macrophages (25). It is known that TNF- α is involved in initiating the inflammatory cascade (3) and that the release of $\cdot\text{NO}$ due to induction of iNOS is part of the inflammatory process. Furthermore, surfactant inhibits the production of TNF- α and $\cdot\text{NO}$ by LPS-stimulated cells. Because surfactant is part of the normal environment for alveolar macrophages in vivo, it is possible that it serves to attenuate some of the inflammatory processes. The local concentration of surfactant around the cells on the alveolar surface is not known, so it is not possible to determine the amount of attenuation that occurs in vivo. All of these findings taken together suggest that surfactant may play a protective role in the lungs by reducing the amount of inflammation and restricting lung damage. This latter idea was suggested by McIntosh et al. (25) with regard to SP-A effects in alveolar macrophages.

Address for reprint requests: P. R. Miles, NIOSH, HELD/MS/2015, 1095 Willowdale Rd., Morgantown, WV 26505.

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