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# Pulmonary Surfactant Protein A Inhibits Macrophage Reactive Oxygen Intermediate Production in Response to Stimuli by Reducing NADPH Oxidase Activity<sup>1</sup>

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Alveolar macrophages are important host defense cells in the human lung that continuously phagocytose environmental and infectious particles that invade the alveolar space. Alveolar macrophages are prototypical alternatively activated macrophages, with up-regulated innate immune receptor expression, down-regulated costimulatory molecule expression, and limited production of reactive oxygen intermediates (ROI) in response to stimuli. Surfactant protein A (SP-A) is an abundant protein in pulmonary surfactant that has been shown to alter several macrophage (M $\phi$ ) immune functions. Data regarding SP-A effects on ROI production are contradictory, and lacking with regard to human M $\phi$ . In this study, we examined the effects of SP-A on the oxidative response of human M $\phi$  to particulate and soluble stimuli using fluorescent and biochemical assays, as well as electron paramagnetic resonance spectroscopy. SP-A significantly reduced M $\phi$  superoxide production in response to the phorbol ester PMA and to serum-opsonized zymosan (OpZy), independent of any effect by SP-A on zymosan phagocytosis. SP-A was not found to scavenge superoxide. We measured M $\phi$  oxygen consumption in response to stimuli using a new oxygen-sensitive electron paramagnetic resonance probe to determine the effects of SP-A on NADPH oxidase activity. SP-A significantly decreased M $\phi$  oxygen consumption in response to PMA and OpZy. Additionally, SP-A reduced the association of NADPH oxidase component p47<sup>phox</sup> with OpZy phagosomes as determined by confocal microscopy, suggesting that SP-A inhibits NADPH oxidase activity by altering oxidase assembly on phagosomal membranes. These data support an anti-inflammatory role for SP-A in pulmonary homeostasis by inhibiting M $\phi$  production of ROI through a reduction in NADPH oxidase activity. *The Journal of Immunology*, 2004, 172: 6866–6874.

The alveolar macrophage (AM $\phi$ )<sup>3</sup> is the predominant front-line innate defense cell in the human lung. AM $\phi$  phagocytose opsonized and nonopsonized microorganisms through a variety of receptors, including complement receptors, Fc $\gamma$ R, the mannose receptor (MR), and various scavenger receptors (1). Following phagocytosis, the microbe-containing phagosome typically fuses with lysosomes, where the ingested mi-

crobe is destroyed by a combination of methods including vesicle acidification, enzymatic digestion, and the oxidative or respiratory burst (2), in which the NADPH oxidase generates reactive oxygen (ROI) and nitrogen intermediates.

ROI and their downstream radical products are highly toxic molecules that control a broad spectrum of infectious agents due to their damaging effects on proteins, RNA, and DNA (3). This antimicrobial mechanism is nonspecific and broad spectrum, and as such is also dangerous for the host. Pulmonary epithelial lining fluid contains variable amounts of enzyme and low m.w. antioxidants to help reduce damage to the underlying tissues (4). However, continuous exposure of pulmonary macrophages to inhaled environmental and infectious particles results in macrophage oxidative responses as well as production of proinflammatory chemokines and cytokines. Recruited neutrophils and monocytes release lytic granule proteins and additional ROI in a positive feedback loop, which, if unchecked, results in unresolved cellular and structural damage to the alveoli and compromised gas exchange.

The lung has an additional mechanism for down-regulating these injurious responses to inhaled stimuli. AM $\phi$  have been shown to be a prototypical alternatively activated macrophage population (5). Alternative activation of macrophages causes the up-regulation of innate immune receptors such as the MR and type I scavenger receptors, and consequently enhances the cell capacity for phagocytosis (6). Even with this increase in phagocytosis, AM $\phi$  have been shown to have a decreased respiratory burst in response to stimuli, compared with blood monocytes (7). This evidence suggests that components within the alveolar space may limit inflammation in the lung by modulating AM $\phi$  production of ROI in response to stimuli.

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<sup>3</sup> Abbreviations used in this paper: AM $\phi$ , alveolar macrophage; Cyt c, cytochrome c; DCF, 2',7'-dichlorodihydrofluorescein; DIC, differential interference contrast; DMPO, 5,5-dimethyl-pyrroline-1-oxide; DOX, 2',7'-dichlorofluorescein diacetate; DPBS, Dulbecco's PBS; DPI, diphenyleneiodonium; EPR, electron paramagnetic resonance; HHG, HEPES + human serum albumin + glucose; HMPS, hexose monophosphate shunt; LiNc-BuO, lithium 5,9,14,18,23,27,32,36-octa-*n*-butoxy-2,3-naphthalocyanine; M $\phi$ , macrophage; MOI, multiplicity of infection; MR, mannose receptor; OpZy, serum-opsonized zymosan; ROI, reactive oxygen intermediate; SOD, superoxide dismutase; SP-A, surfactant protein A.

Pulmonary surfactant is a complex mixture of lipids and proteins that reduces surface tension at the air-liquid interface within the alveoli. Resident AM $\phi$  are bathed in surfactant and ingest abundant amounts of this material (8). Therefore, the role of surfactant components in modulating macrophage antibacterial function has been of great interest (9). The most abundant protein component of alveolar surfactant (by weight) is surfactant protein A (SP-A). This ~600-kDa protein, produced by type II alveolar epithelial cells, is a member of the collectin protein family, which also contains innate immune proteins such as complement component C1q and mannose-binding lectin. Purified SP-A has been shown to alter several macrophage immune functions in vitro, including chemotaxis, phagocytosis, cytokine production, and reactive nitrogen intermediate production (9, 10).

There is limited literature regarding the effects of SP-A on macrophage ROI production. SP-A itself has been shown to induce superoxide production in rat alveolar macrophages (11), and to either increase (11, 12) or decrease ROI production in response to agonists in rat and canine alveolar macrophages (13, 14). Thus, while the data are not easily reconciled, they do suggest a role for SP-A in regulating the macrophage oxidative burst.

The aim of the present study was to test the hypothesis that SP-A reduces human macrophage production of ROI through the NADPH oxidase in response to stimuli. Using several complementary approaches, we examined the respiratory burst of human monocyte-derived macrophages in response to the phagocytic stimulus serum-opsonized zymosan (OpZy) and the chemical stimulus PMA in the presence or absence of purified human SP-A. Our results demonstrate a reduction of macrophage ROI production by SP-A in response to both stimuli, and indicate for the first time that SP-A exerts its effects by reducing the activity of the macrophage NADPH oxidase, and not by scavenging of ROI.

## Materials and Methods

### Proteins and chemical reagents

Zymosan A (Sigma-Aldrich, St. Louis, MO) was opsonized for 30 min at 37°C in 50% human serum, then washed at 4°C with protease inhibitors leupeptin, pepstatin, aprotinin, and soybean trypsin inhibitor. Protease inhibitors, 2',7'-dichlorofluorescein diacetate (DOX), cytochrome *c* (CytC), catalase, superoxide dismutase (SOD), BSA, diphenyleneiodonium (DPI), and PMA were obtained from Sigma-Aldrich. The 2',7'-dichlorodihydrofluorescein diacetate (DCF) and Alexa Fluor 546 donkey anti-goat Abs were purchased from Molecular Probes (Eugene, OR). Cell culture medium was obtained from Life Technologies/Invitrogen (Carlsbad, CA). Human serum albumin (25% solution) was obtained through University of Iowa Pharmacy Services. The 5,5-dimethyl-pyrroline-1-oxide (DMPO) was purchased from Dojindo (Kumamoto, Japan). Goat anti-p47<sup>phox</sup> polyclonal Ab and normal goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### SP-A purification

SP-A was purified, as reported previously (15), with modifications. Briefly, bronchial lavage fluid from human alveolar proteinosis patients, who overproduce surfactant, was centrifuged at 20,000  $\times$  *g*, washed repeatedly, and eluted using 2 mM EDTA at 4°C. SP-A was purified from the supernatant by separation over a mannose-Sepharose affinity chromatography column, and EDTA was removed by dialysis against 10 mM HEPES + 25 mM NaCl. All steps were performed under sterile conditions at 4°C whenever possible. Endotoxin-free water (Baxter, Round Lake, IL) was used for all steps to reduce LPS contamination. Purified protein was examined by SDS-PAGE and Western blot using rabbit anti-SP-A antiserum, and endotoxin contamination was determined using a *Limulus* Amoebocyte Lysate assay kit with *Escherichia coli* LPS standards (BioWhittaker/Cambrex, East Rutherford, NJ). Endotoxin concentration was  $\leq 0.25$  pg/ $\mu$ g protein.

### Cell preparation

Human PBMC were isolated from heparinized peripheral blood of healthy donors using Ficoll-Paque (Amersham, Piscataway, NJ) and cultured in Teflon wells (Savillex, Minnetonka, MN) in RPMI 1640 + 20% autolo-

gous serum for 5–6 days at 37°C, 5% CO<sub>2</sub>. Monocyte-derived macrophages (M $\phi$ ) were isolated from cultured PBMC by adhering to tissue culture plates (BD Falcon, Bedford, MA) for 2 h at 37°C in 10% autologous serum, washed, and replated with Dulbecco's PBS + 10 mM HEPES + 1 mg/ml human serum albumin + 0.1% glucose (DPBS-HHG). Cell viability was determined by trypan blue exclusion and was  $\geq 90\%$ .

### DCF assay for detecting the respiratory burst

On the day of each experiment, M $\phi$  were adhered to 96-well plates at 3E6 PBMC/ml (~6E4 M $\phi$ /well), as above, and replated in DPBS-HHG + 32 mM DCF for 30 min at 37°C. For SP-A preincubation conditions, M $\phi$  were incubated with 10  $\mu$ g/ml SP-A for 30 min. Cells were then stimulated with OpZy (multiplicity of infection (MOI) 12:1), PMA (1  $\mu$ g/ml), SP-A (10  $\mu$ g/ml), and/or medium-only control. For conditions of synchronized phagocytosis, cells were cooled to 4°C for 10 min before addition of stimuli, and OpZy were centrifuged onto M $\phi$  at 200  $\times$  *g* for 10 min. Cells were then warmed to 37°C, and fluorescence of DCF, indicative of ROI production, was measured every 2 min for a total of 90 min using a Fluostar32 plate reader (BMG Lab Technologies, Durham, NC). Test conditions were measured in triplicate wells, and best-fit rate curves were determined using SigmaPlot 5.0 (SPSS, Chicago, IL). Single time-point ROI levels were calculated at 60 min by setting all values relative to unstimulated cell controls.

### Fluorescence assay for DCF quenching

DCF and its oxidized (fluorescent) counterpart DOX were hydrolyzed with 10 mM NaOH for 30 min in the dark (16), then diluted in DPBS-HHG. DOX and DCF were incubated in a dose titration of 6 nM–6  $\mu$ M with 10  $\mu$ g/ml SP-A in triplicate in a 96-well plate, and well fluorescence was measured every 5 min for a total of 45 min using a Fluostar32 plate reader. Fluorescence of DOX was above background (i.e., greater than DCF) at doses of 600 nM and greater.

### Phagocytosis assay

M $\phi$  were adhered to acid-cleaned glass coverslips (~1.5E5 M $\phi$ /well) in 24-well plates and replated in DPBS-HHG, as above. Cells were incubated with 10  $\mu$ g/ml SP-A and/or OpZy (MOI 10:1) for 10 or 30 min at 37°C, washed, and fixed in 2% paraformaldehyde. The number of particles per cell on duplicate or triplicate coverslips was determined by counting a minimum of 50 consecutive cells per coverslip using phase contrast microscopy.

### CytC assay for detecting superoxide production

M $\phi$  were adhered to 24-well plates at 3E6 PBMC/ml (~1.5E5 M $\phi$ /well), as above, and replated in DPBS-HHG. For conditions of synchronized phagocytosis, cells were cooled to 4°C for 10 min before addition of stimuli, and OpZy were centrifuged onto M $\phi$  at 200  $\times$  *g* for 10 min. Cells were stimulated with OpZy (MOI 12:1), PMA (1  $\mu$ g/ml), SP-A (20  $\mu$ g/ml), and/or medium-only control in triplicate wells in the presence of 80  $\mu$ M CytC and 500 U/ml catalase at 37°C for 60 min. CytC reduction indicative of superoxide production was measured by subtracting the absorbance at 550 nm of control wells treated with 300 U/ml SOD from test well values. Unstimulated cell background values were subtracted from test conditions, and all values were set relative to the positive control.

### Electron paramagnetic resonance (EPR) assay for detecting oxidant production

Cells were adhered to four-well plates, as above, washed, and rested overnight in RPMI 1640 + 10% serum. M $\phi$  were lifted using rubber policeman and ice-cold DPBS-HHG, resuspended in DPBS-HHG, and kept on ice until used. Cells in suspension at 6E6/ml were stimulated with mock, PMA (400 ng/ml), OpZy (MOI 1:1), SP-A (20  $\mu$ g/ml), and/or SOD (100 U/ml), and free radical generation was measured over 30 min using the spin trap DMPO (50 mM). No background DMPO adduct formation was observed in unstimulated cells. All values were set relative to positive controls.

EPR measurements were performed using a Bruker X-band (9.8 GHz) EPR spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with a TM110 cavity. A quartz flat cell was used as the sample holder. Spectra were recorded at room temperature (22–25°C) using data acquisition software (SPEX) capable of fully automated data acquisition and processing. Quantification of the observed DMPO adduct was performed using a simulation-fitting procedure, as described (17). In short, the experimental spectra of the spin adducts were precisely simulated using multicomponent fitting, and the double integral (area) of the simulated spectra was compared with that of a standard sample using 2,2,6,6-tetramethylpiperidine-1-oxyl (1  $\mu$ M) measured under identical conditions. We have previously

established that the quantification by the simulated-fitting procedure is accurate and reliable (17).

#### EPR assay for detecting superoxide scavenging

Superoxide was generated in DPBS using the reaction of xanthine (500  $\mu$ M) and xanthine oxidase (0.02 U/ml) in the presence of 0–500  $\mu$ g/ml SP-A or 100 U/ml SOD control. Superoxide levels were measured by EPR using the spin trap DMPO ( $n = 3$ ) and expressed as mean percentage of negative (no inhibitor) control.

#### CytC assay for detecting superoxide scavenging

Superoxide was generated in reaction buffer (20 mM  $\text{Na}_2\text{CO}_3 + 0.1$  mM EDTA + 1.0 mM  $\text{NaN}_3 + 5$   $\mu$ M CytC) using the reaction of xanthine (50  $\mu$ M) and xanthine oxidase (0.025 U/ml) in the presence of 0–100  $\mu$ g/ml SP-A or 0.1 U/ml SOD in triplicate wells. The change in SOD-inhibitable absorbance at 550 nm, indicative of superoxide generation, was determined after 5 min at 37°C. Background values, obtained by measuring the absorbance of wells that did not contain xanthine oxidase, were subtracted from all test conditions.

#### EPR assay for detecting oxygen consumption

Cells were adhered to four-well plates, as above, washed, and rested overnight in RPMI 1640 + 10% serum. M $\phi$  were lifted using rubber policeman and ice-cold DPBS-HHG, resuspended in DPBS-HHG, and kept on ice until used. Cells in suspension at 6E6/ml were treated with potassium cyanide (KCN) (100  $\mu$ M) to inhibit mitochondrial oxygen consumption and/or DPI (100  $\mu$ M) to inhibit total oxygen consumption and stimulated with mock, PMA (400 ng/ml), or OpZy (MOI 1:1), in the presence or absence of 20  $\mu$ g/ml SP-A for 30 min. Oxygen concentration in the reaction mixture over time was measured in sealed capillary tubes, with a minimum of two tubes per condition, by EPR oximetry using the oxygen-sensitive probe lithium 5,9,14,18,23,27,32,36-octa-*n*-butoxy-2,3-naphthalocyanine (LiNc-BuO), whose line width and shape in EPR correspond to the  $p\text{O}_2$  in the reaction (18). The data of measured peak-to-peak line width were converted to  $p\text{O}_2$  using a standard curve of line width vs  $p\text{O}_2$  prepared by equilibrating the oximetry probe with known concentrations of oxygen, as reported (19). The rate (R) of oxygen consumption in nmol/min/ $10^6$  cells was determined over time using the equation  $R = m\alpha$ , where  $m$  is the slope of the  $p\text{O}_2$  curve (in  $p\text{O}_2/\text{min}$ ) determined by linear regression, and  $\alpha$  is the solubility of oxygen in water (1.59 nmol/mmHg at 22°C). Unstimulated cell oxygen consumption rates were subtracted from each test condition, and data were expressed as percentage of positive control.

#### Colocalization experiments for NADPH oxidase assembly

M $\phi$  were adhered to acid-cleaned glass coverslips in 24-well plates at 4E6 cells/ml (~2E5 M $\phi$ /well), as above, and replated in DPBS-HHG. M $\phi$  were incubated with 10  $\mu$ g/ml SP-A for 30 min and cooled to 4°C for 10 min before addition of stimuli, and OpZy (MOI 4:1) were centrifuged onto M $\phi$  at  $200 \times g$  for 10 min. Cells were then warmed to 37°C for 5 min, washed, fixed in 2% paraformaldehyde, and permeabilized in 100% methanol. Coverslips were blocked in 0.2% BSA overnight at 4°C, and then stained with 4  $\mu$ g/ml goat anti-p47<sup>phox</sup> polyclonal Ab or normal goat IgG control for 1 h at room temperature, followed by Alexa Fluor 546 donkey anti-goat IgG (1/250). Coverslips were washed and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined by confocal microscopy.

#### Confocal microscopy

Slides were examined using a  $\times 63$  oil-immersion objective on a Zeiss (Oberkochen, Germany) 510 META laser scanning confocal microscope. Cell-associated zymosan were identified using differential interference contrast (DIC), and the percentage of positive zymosan was determined for each condition by counting a minimum of 40–200 (average 118) zymosan per coverslip on triplicate coverslips.

#### Statistics

The numbers of experiments cited are independent experiments performed on separate occasions with a minimum of two different donors. A two-tailed Student's  $t$  test was used to analyze differences between test groups, except where a test group result was expressed as a relative value and then analyzed with respect to the control, in which case a one-sample  $t$  test was used to determine statistical significance. Differences between groups were considered significant at  $p < 0.05$ .

## Results

### SP-A reduces the respiratory burst in response to phagocytic and chemical stimuli

To examine the effect of SP-A on the respiratory burst of human macrophages, we measured the response of adherent human peripheral blood monocyte-derived M $\phi$  to OpZy in the presence or absence of 10  $\mu$ g/ml human SP-A using the fluorescent probe DCF. Fluorescence, indicative of ROI production, was measured over 90 min. Baseline ROI production was determined by measuring the fluorescence of control cells treated with medium only. SP-A alone did not induce production of ROI by M $\phi$ . In early experiments, SP-A significantly reduced the initial rate of M $\phi$  ROI production by  $63 \pm 3\%$  (mean  $\pm$  SEM,  $n = 11$ ,  $p < 0.0001$ ) when simultaneously added to the M $\phi$  with OpZy, and reduced the production rate by  $57 \pm 5\%$  (mean  $\pm$  SEM,  $n = 8$ ,  $p < 0.0001$ ) when M $\phi$  were incubated with SP-A 30 min before stimulation with OpZy.

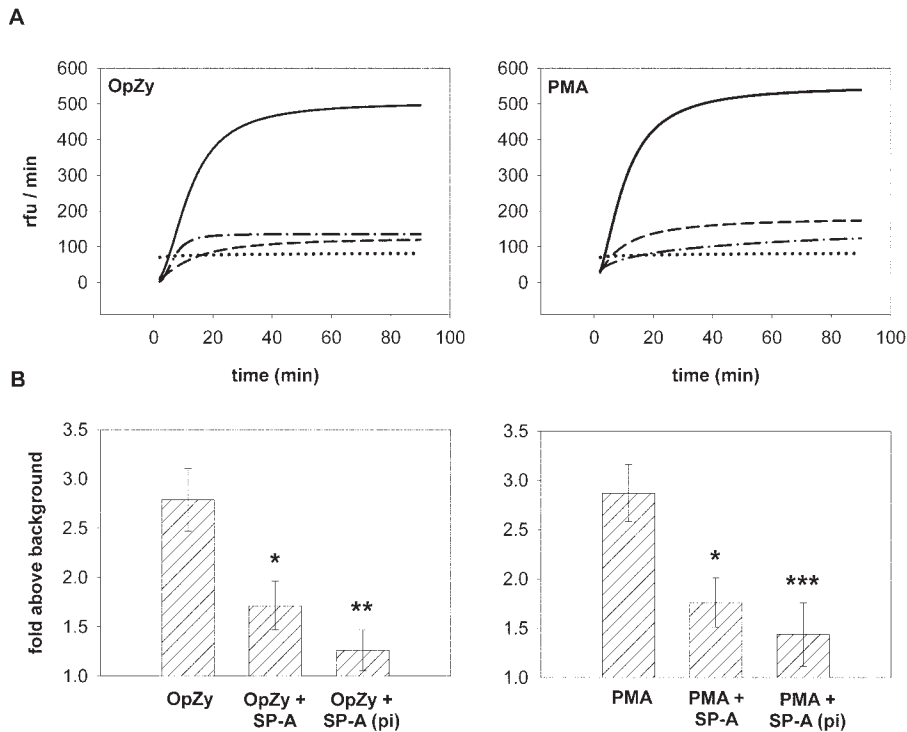
In the above experiments, zymosan was added to the cell medium at 37°C. Under these conditions, initiation of the respiratory burst due to OpZy receptor binding and ingestion occurs randomly over the course of the assay, as the zymosan particles encounter the M $\phi$  monolayer. To better examine the effects of SP-A on M $\phi$  ROI production over time, we next synchronized phagocytosis in our assays by centrifuging zymosan onto the M $\phi$  monolayer at 4°C, which allows receptor binding, but prevents ingestion and ROI production. The cells were then warmed to 37°C, enabling ROI production in response to phagocytic events occurring simultaneously. Under these conditions, there was a more striking reduction in the rate of ROI production by SP-A; at 60 min, ROI levels in response to OpZy were significantly reduced by both simultaneous SP-A addition and pretreatment of M $\phi$  with SP-A compared with stimulation with OpZy alone (Fig. 1).

SP-A has been demonstrated to affect the phagocytosis of a variety of organisms (9, 10). To determine whether inhibition of M $\phi$  ROI by SP-A was due to a reduction in macrophage phagocytosis of OpZy, adherent human M $\phi$  were incubated with OpZy (12:1) in the presence of 10  $\mu$ g/ml SP-A or medium control for 30 min and fixed, and the number of zymosan per cell was determined by counting under phase contrast microscopy. SP-A did not reduce M $\phi$  phagocytosis of OpZy (SP-A treated vs mock,  $6.73 \pm 0.54$  and  $6.93 \pm 0.24$  particles per cell,  $n = 2$ ,  $p > 0.6$ ). Therefore, we concluded that the effect of SP-A on the M $\phi$  respiratory burst was independent of an effect on M $\phi$  phagocytosis. To further confirm this observation, we used DCF to measure M $\phi$  ROI in response to the receptor-independent chemical stimulus PMA. SP-A caused a significant inhibition of the respiratory burst induced by PMA treatment of M $\phi$  monolayers (Fig. 1).

It was conceivable that the inhibition of ROI seen with SP-A in the DCF assay was due to nonspecific quenching of DCF fluorescence by SP-A (i.e., technical artifact). In cell-free assays, oxidized DCF fluorescence was not reduced by 10  $\mu$ g/ml SP-A at 600 nM–6  $\mu$ M DCF ( $n = 3$ ), indicating that SP-A inhibition of DCF fluorescence in stimulated M $\phi$  is due to an effect of SP-A on ROI and not an interaction of SP-A with the probe.

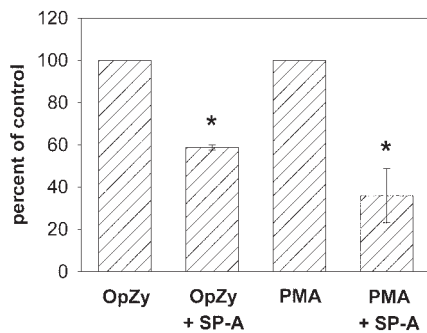
To further examine the inhibition of M $\phi$  ROI by SP-A, we next assessed superoxide production in mock- or SP-A-treated M $\phi$  in response to OpZy (synchronized phagocytosis) or PMA after 60-min stimulation by measuring the SOD-inhibitable reduction of exogenous CytC. SP-A reduced the level of M $\phi$  superoxide by  $64 \pm 12.7\%$  in response to PMA and  $42 \pm 1.3\%$  in response to OpZy (Fig. 2).

As a more sensitive and direct quantitative approach to examining the reduction of the M $\phi$  oxidative response by SP-A, we



**FIGURE 1.** SP-A reduces macrophage ROI generation in response to stimuli. M $\phi$  were adhered to 96-well plates at 3E6 PBMC/ml ( $\sim 6E4$  M $\phi$ /well) and replated in DPBS-HHG + 32 mM DCF for 30 min at 37°C. For SP-A preincubation (pi) conditions, M $\phi$  were incubated with 10  $\mu$ g/ml SP-A for 30 min. Cells were treated with OpZy (12:1), PMA (1  $\mu$ g/ml), SP-A (10  $\mu$ g/ml), and/or medium-only control. To synchronize phagocytosis, cells were cooled to 4°C for 10 min before addition of stimuli, and OpZy were centrifuged onto M $\phi$  at 200  $\times$  g for 10 min. Cells were then warmed to 37°C, and fluorescence of DCF, indicative of ROI production, was measured every 2 min over 90 min using a Fluostar32 plate reader. *A*, Rate of ROI production over time in relative fluorescence units (rfu) per minute for (.....), no stimulus; (—), OpZy or PMA (*left* and *right* panels, respectively); (— — —), OpZy or PMA, + SP-A; (— — —), OpZy or PMA, + SP-A (pi). Representative of three to eight independent experiments. *B*, ROI values at 60 min in response to indicated stimuli, expressed as mean fluorescence  $\pm$  SEM relative to unstimulated cell controls (value = 1.0) for 5–16 independent experiments. \*,  $p < 0.02$ ; \*\*,  $p < 0.005$ ; \*\*\*,  $p < 0.01$ , compared with cells stimulated with OpZy or PMA alone.

analyzed the effect of SP-A on macrophage ROI generation in response to PMA and OpZy using EPR spectroscopy with the spin trap DMPO. This method allows simultaneous detection of super-

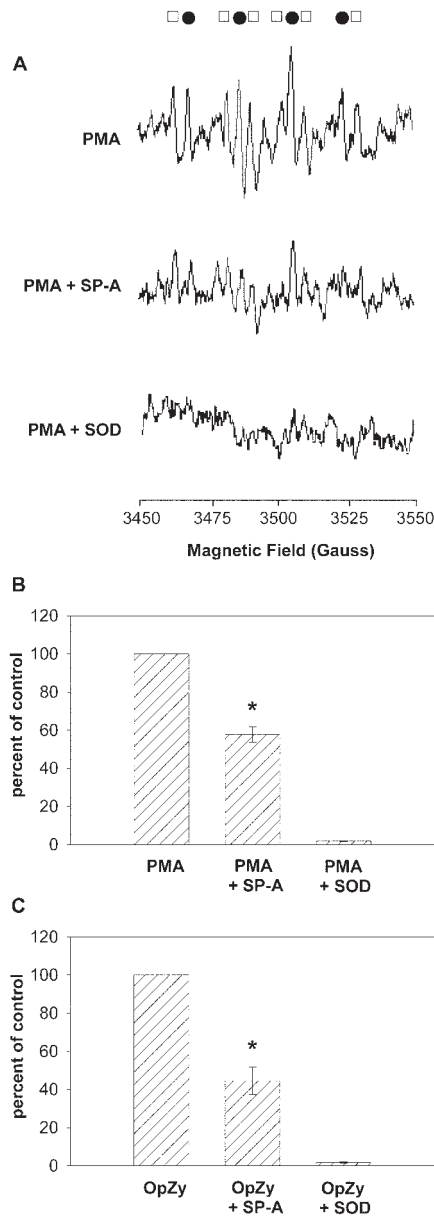


**FIGURE 2.** SP-A reduces macrophage superoxide generation in response to stimuli. M $\phi$  were adhered to 24-well plates at 3E6 cells/ml ( $\sim 1.5E5$  M $\phi$ /well) and replated in DPBS-HHG. Cells were treated with OpZy (12:1), PMA (1  $\mu$ g/ml), SP-A (20  $\mu$ g/ml), and/or medium-only control in triplicate wells in the presence of 500 U/ml catalase and 80  $\mu$ M CytC. To synchronize phagocytosis, cells were cooled to 4°C for 10 min before addition of stimuli, and OpZy were centrifuged onto M $\phi$  at 200  $\times$  g for 10 min. CytC reduction, indicative of superoxide production, was measured after 60 min of stimulation by subtracting the absorbance at 550 nm of control wells treated with 300 U/ml SOD from test well values. Unstimulated cell background values were subtracted from test conditions, and all values were set relative to the positive control. Results are expressed as percentage of OpZy- or PMA-stimulated control  $\pm$  SEM for two to four independent experiments. \*,  $p < 0.02$ , compared with cells stimulated with OpZy or PMA alone.

oxide and its downstream ROI, hydroxyl- and carbon-centered radicals (20). Fig. 3A shows representative EPR spectra obtained in M $\phi$  stimulated with PMA. No superoxide was detected in response to PMA or OpZy in these assays due to the short  $t_{1/2}$  of the DMPO-superoxide adduct and the low levels of superoxide produced by human macrophages. However, stimulated macrophages did produce detectable free radical DMPO adducts of hydroxyl and carbon-centered radicals. The origin of these radicals was superoxide, because incubation of PMA- or OpZy-stimulated macrophages with SOD almost completely abrogated the DMPO-adduct signals (Fig. 3A). SP-A treatment of macrophages significantly reduced the free radical production in response to both PMA and OpZy (Fig. 3, B and C). Together, these data provide strong evidence that SP-A reduces the macrophage respiratory burst in response to stimuli.

#### SP-A does not scavenge superoxide

Since the preceding techniques only measure the direct or indirect products of the respiratory burst, they cannot differentiate between changes in detectable ROI levels due to altered NADPH oxidase activity and those due to scavenging of the ROI produced. Therefore, to examine whether SP-A decreased macrophage ROI in response to stimuli by scavenging superoxide, we generated superoxide in a cell-free system through the xanthine/xanthine oxidase enzymatic reaction in the presence of 0–500  $\mu$ g/ml SP-A and measured the amount of superoxide using EPR with the spin trap DMPO. SOD inhibited superoxide detected by DMPO by 98.5  $\pm$  0.3% in our controls. However, no reduction in superoxide levels was observed in the presence of 10, 100, or 500  $\mu$ g/ml SP-A (data



**FIGURE 3.** SP-A reduces free radical formation by macrophages in response to stimuli. Cells were adhered to four-well tissue culture plates, washed, and rested overnight in RPMI 1640 + 10% serum. M $\phi$  were lifted using rubber policemen and ice-cold DPBS-HHG, resuspended in DPBS-HHG, and kept on ice until used. Cells in suspension at 6E6/ml were stimulated with PMA (400 ng/ml), OpZy (1:1), SP-A (20  $\mu$ g/ml), and/or SOD (100 U/ml), and free radical generation was measured over 30 min using the spin trap DMPO. *A*, Representative spectra showing characteristic  $\cdot$ OH ( $\bullet$ ) and  $\cdot$ R ( $\square$ ) peaks for each condition. *B* and *C*, Quantitation of free radical production at 30 min for PMA (*B*) and OpZy (*C*). Results are expressed as percentage of PMA- or OpZy-induced DMPO adduct formation  $\pm$  SD and are representative of two to four experiments. \*,  $p < 0.002$ , compared with cells stimulated with PMA or OpZy alone.

not shown,  $n = 4$ ). These studies were also performed using the reduction of CytC to measure the amount of superoxide produced by the xanthine/xanthine oxidase reaction in the presence of 0–100  $\mu$ g/ml SP-A, with identical results ( $n = 3$ ). These results indicate SP-A is not a scavenger of superoxide.

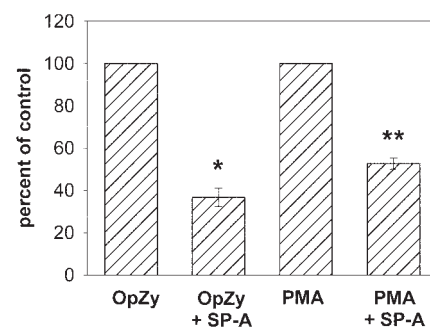
#### SP-A reduces NADPH oxidase activity

Since the above results provided evidence that the decrease in M $\phi$  ROI by SP-A was not due to scavenging of superoxide, we hy-

pothesized that SP-A inhibited the oxidative burst in response to stimuli by reducing the activity of the M $\phi$  NADPH oxidase. Since oxygen is the central component consumed in the production of ROI, we measured the rate of oxygen consumption by M $\phi$  over time using EPR oximetry with the oxygen-sensitive probe LiNc-BuO, whose spectral line width and shape in EPR correspond to the pO $_2$  in the reaction medium (18). SP-A alone did not reduce the oxygen consumption of resting M $\phi$  (data not shown). Oxygen consumption in the stimulated macrophage is due to both mitochondrial respiration and agonist-induced NADPH oxidase activity; we therefore used 100  $\mu$ M KCN to inhibit mitochondrial oxygen consumption by the macrophage. KCN had no inhibitory effect on the increased oxygen consumption rate in response to PMA, indicating the observed increase was not due to increased mitochondrial respiration, while DPI, an inhibitor of both mitochondrial and NADPH oxidase respiration, completely inhibited the increase in oxygen consumption rate in response to PMA. Thus, an increase in the rate of oxygen consumption in the presence of KCN was expected to be due primarily to NADPH oxidase activity. Treatment of M $\phi$  with SP-A reduced oxygen consumption by  $47 \pm 5\%$  in response to PMA (mean  $\pm$  SEM,  $n = 3$ ,  $p < 0.005$ ) and  $63 \pm 4\%$  in response to OpZy (mean  $\pm$  SEM,  $n = 2$ ,  $p < 0.05$ ) (Fig. 4). Therefore, we concluded that SP-A reduces the macrophage oxidative burst in response to stimuli by inhibiting the activity of the NADPH oxidase.

#### SP-A reduces colocalization of p47<sup>phox</sup> with OpZy phagosomes

Studies in neutrophils from normal and chronic granulomatous disease patients have shown that assembly of a functional NADPH oxidase requires the translocation of cytosolic NADPH oxidase component p47<sup>phox</sup> to the membrane component cytochrome b<sub>558</sub> (21, 22). To investigate the mechanism(s) by which SP-A inhibits NADPH oxidase activity, we therefore examined colocalization of p47<sup>phox</sup> with OpZy phagosomes. M $\phi$  adhered to glass coverslips



**FIGURE 4.** SP-A reduces oxygen consumption through the NADPH oxidase in response to stimuli. Cells were adhered to four-well plates, washed, and rested overnight in RPMI 1640 + 10% serum. M $\phi$  were lifted using rubber policemen and ice-cold DPBS-HHG, resuspended in DPBS-HHG, and kept on ice until used. Cells in suspension at 6E6/ml were treated with KCN (100  $\mu$ M) to inhibit mitochondrial oxygen consumption and stimulated with 400 ng/ml PMA or OpZy (1:1), in the presence or absence of 20  $\mu$ g/ml SP-A for 30 min. Oxygen concentration in the reaction mixture over time was measured in sealed capillary tubes, with a minimum of two replicate tubes per condition, using EPR oximetry with the oxygen-sensitive probe LiNc-BuO. Unstimulated cell oxygen consumption rates were subtracted from each test condition. Results are expressed as percentage of positive control  $\pm$  SEM for two to three independent experiments. \*,  $p < 0.05$ , compared with cells stimulated with OpZy alone; \*\*,  $p < 0.005$ , compared with cells stimulated with PMA alone. Basal oxygen consumption rates varied by donor. Rates in unstimulated M $\phi$  treated with medium alone vs KCN alone were 0.2–0.4 nmol/min/1E6 cells and 0.03–0.2 nmol/min/1E6 cells, respectively.

were incubated with 20  $\mu\text{g/ml}$  SP-A for 30 min and then stimulated with OpZy under conditions of synchronized phagocytosis for 5 min. Cell-associated OpZy were visualized using DIC, and phagosome-associated p47<sup>phox</sup> was detected using immunofluorescent staining and confocal microscopy. SP-A treatment of M $\phi$  reduced the number of p47<sup>phox</sup>-positive OpZy phagosomes by  $38.1 \pm 7.5\%$  (mean  $\pm$  SEM,  $n = 3$ ,  $p < 0.04$ ). Additionally, the intensity of p47<sup>phox</sup> staining on positive OpZy phagosomes was decreased in SP-A-treated cells (Fig. 5). These data suggest a mechanism for SP-A reduction in NADPH oxidase activity is reduction of p47<sup>phox</sup> association with the phagosome.

## Discussion

In the healthy lung, the AM $\phi$  represents the first line of cellular innate defense against environmental particles and invading microorganisms that reach the lower airways. These cells are highly phagocytic and express a broad range of immune receptors, including Fc receptors and complement receptors (23) as well as pattern recognition receptors such as the MR (24) and Toll-like receptors (25). To combat the constant stimulation by inhaled particles and microbes, the AM $\phi$  displays an anti-inflammatory phenotype, which includes altered cytokine production and a reduced oxidative response to stimuli (1).

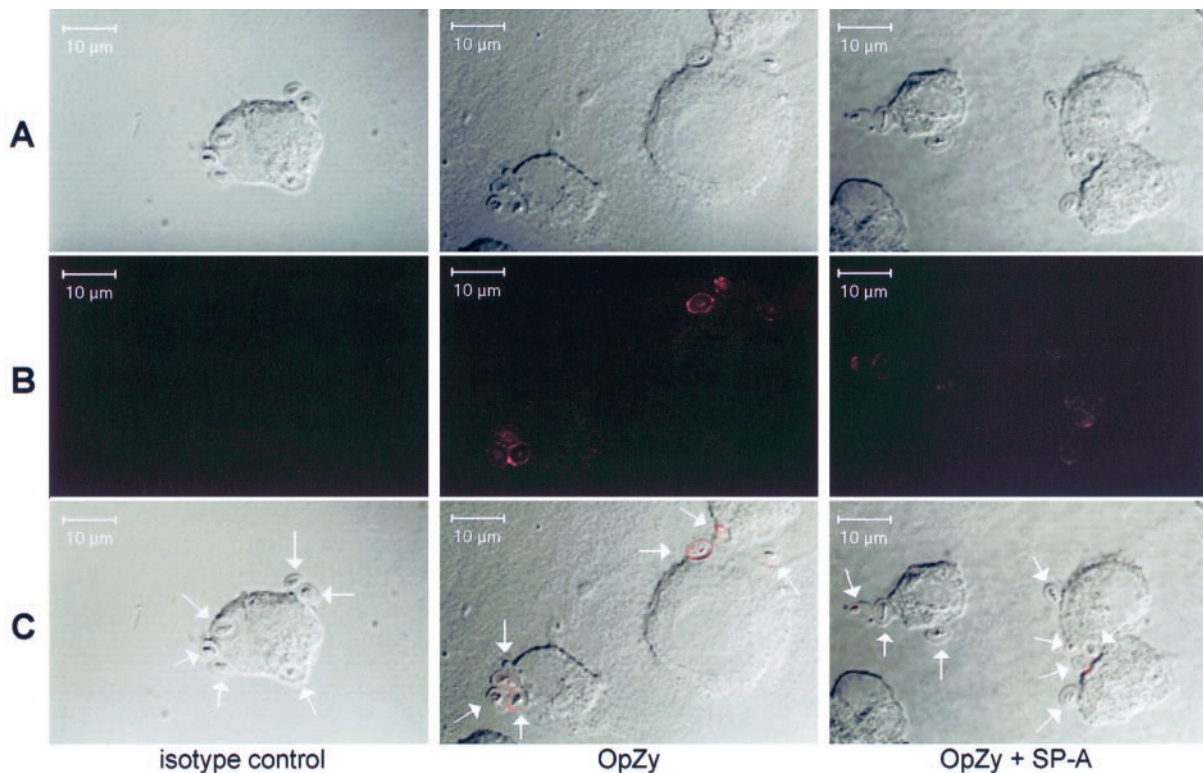
There has been much interest about what induces this alternatively activated state in AM $\phi$ . AM $\phi$  are thought to originate both from circulating monocytes and from interstitial monocytes/macrophages that transmigrate into the alveolar environment, where they encounter molecules that influence their maturation (1). Data are accumulating that indicate components of pulmo-

nary surfactant play important roles in host lung defense, both as innate defense proteins and as modulators of cellular immune activities (9, 10).

The predominant protein component of surfactant (by weight) is the surfactant-associated collectin SP-A. Binding of SP-A to macrophages has been described (26), and its reported effects on multiple phenotypic and functional aspects of macrophage biology include up-regulation of MR activity (27), increased phagocytosis through FcR and complement receptor 1 (28), altered production of proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$  (9, 10), and decreased production of NO in response to stimuli (9, 10).

Although whole surfactant has been shown to inhibit macrophage oxidative responses (29), the contributory effects of SP-A on macrophage ROI production are not clear. Katsura et al. (13) demonstrated a dose-dependent inhibition by SP-A of rat AM $\phi$  superoxide production in response to both PMA and zymosan, and Weber et al. (14) showed that SP-A inhibited the respiratory burst in canine AM $\phi$  by 50% in response to the chemical stimulus PMA. In contrast, van Iwaarden et al. (11) reported that human SP-A treatment of rat AM $\phi$  increased the oxidative response to opsonized *Staphylococcus aureus*. Their findings were supported by the work of Weissbach et al. (12), which described a dose-dependent enhancement of the rat AM $\phi$  oxidative response to SP-A-opsonized zymosan compared with unopsonized zymosan. Additionally, SP-A may not be the sole effector in surfactant, because some surfactant phospholipids have also been shown to have an inhibitory effect on the respiratory burst (30, 31).

In this study, we show that SP-A treatment of human macrophages significantly reduces their production of superoxide and



**FIGURE 5.** SP-A reduces colocalization of p47<sup>phox</sup> with OpZy phagosomes. Cells were adhered to glass coverslips in 24-well plates at 4E6 cells/ml ( $\sim 2 \times 10^5$  M $\phi$ /well), washed, and replated in DPBS-HHG. Cells were preincubated with SP-A (20  $\mu\text{g/ml}$ ) for 30 min at 37°C. To synchronize phagocytosis, cells were cooled to 4°C for 10 min before addition of stimuli, and OpZy (4:1) were centrifuged on to M $\phi$  at  $200 \times g$  for 10 min. M $\phi$  were incubated at 37°C for 5 min, washed, then fixed in 2% paraformaldehyde and permeabilized in methanol. Coverslips were blocked overnight at 4°C, and then stained with goat anti-p47<sup>phox</sup> polyclonal Ab or normal goat IgG control, followed by Alexa Fluor 546 donkey anti-goat IgG. Cells were visualized using confocal microscopy. Images shown are A, DIC; B, p47<sup>phox</sup> (red); and C, merged for isotype control, OpZy, and OpZy + SP-A, and are representative of three independent experiments. Arrows indicate cell-associated OpZy. Bar equals 10  $\mu\text{m}$ .

downstream ROI in response to both OpZy and PMA. Importantly, our data provide the first evidence that the effect of SP-A on ROI production is not due to scavenging, but rather a reduction in the activity of the NADPH oxidase.

The difference in our results compared with previous reports of SP-A stimulation of ROI may be due to endotoxin (LPS) contamination of the SP-A preparations used. LPS can act both as a stimulatory and an inhibitory molecule, depending on the experimental paradigm. SP-A purified from the lung lavage of humans and animals contains variable levels of endotoxin. Wright et al. (32) have demonstrated that SP-A induction of NO production by rat AM $\phi$  is related to the level of endotoxin contamination in the SP-A preparation. In their studies, LPS contamination above 20 pg/ $\mu$ g SP-A induced significant nitrite production, while endotoxin concentrations below this had no effect. The endotoxin contamination in our SP-A preparations was generally <0.25 pg/ $\mu$ g SP-A, and SP-A alone did not stimulate ROI production by human macrophages in our system. Second, the differences between our results and those of other investigators may be partially due to species differences: Rat AM $\phi$  were used to demonstrate the generation and enhancement of the respiratory burst by human or rat SP-A (11, 12). Our report is the first to demonstrate an effect of SP-A on macrophages of human origin. These differences cannot completely be attributed to differences in rat and human macrophage biology, however, because Katsura et al. (13) also used rat macrophages to demonstrate the inhibitory effect of SP-A on ROI. The activation state of the cell may play an important role in the effects of SP-A as well. We found no effect by SP-A on resting macrophage ROI production, which agrees with the experiments of van Iwaarden et al. (11) that found no stimulatory effect of SP-A in resting human monocytes. Therefore, it is likely that multiple factors contributed to the observed differences between these studies.

We used three different assays to examine the respiratory burst in our system: DCF, a H<sub>2</sub>O<sub>2</sub>-dependent fluorescent probe (16); CytC, whose reduction by superoxide causes a change in protein absorbance at 550 nm (33); and EPR with the spin trap DMPO, which measures multiple species of ROI through spectral differences in the adducts formed by the reaction of DMPO with ROI (20). The use of complementary assays to study the respiratory burst was necessary because of the known limitations in sensitivity and specificity of each assay type. DCF detects H<sub>2</sub>O<sub>2</sub> in a peroxidase-dependent manner; however, the probe is not highly specific for H<sub>2</sub>O<sub>2</sub>, as it can also be oxidized by ONOO<sup>-</sup> (34) as well as some proteins (16). The CytC assay detects extracellular superoxide by measuring the SOD-inhibitable reduction of CytC (33); however, hydrogen peroxide formed by the spontaneous or enzymatic dismutation of superoxide may reoxidize reduced CytC, causing an underestimation of superoxide production (35). We therefore performed these assays in the presence of catalase to limit this artifact. Finally, spin trapping with DMPO by EPR can detect and discriminate between superoxide, hydroxyl, and carbon-centered radicals (20).

The goal of the AM $\phi$  is first to prevent infection of the host by phagocytosing and killing invading microorganisms. Although the AM $\phi$  is equipped with several nonoxidative antibacterial mechanisms (1), its ability to eliminate inhaled microorganisms is significantly impaired if no reactive oxygen species are formed, such as is seen in chronic granulomatous disease (36). The cumulative results from our assays indicate macrophage ROI production in response to stimulation is significantly reduced, but not abolished in the presence of SP-A. These data support the concept that in the lung there is a balance in ROI production between promoting host defense and avoiding host damage.

We found no inhibitory effect of SP-A on phagocytosis of zymosan; therefore, we concluded that SP-A did not reduce ROI levels by reducing phagocytosis. Additionally, SP-A inhibited the macrophage response to the chemical stimulus PMA, which directly activates protein kinase C-driven oxidase responses (37) and therefore bypasses the engagement of a surface receptor. This demonstrated that the inhibitory effect of SP-A on the macrophage respiratory response was independent of an effect on phagocytosis or phagocytic receptors. Respiratory burst assays only measure detectable ROI, which may or may not be equal to the total amount of ROI produced. If a scavenger of reactive oxygen specie(s) is present, there will be a reduction in the amount of that ROI that is detected, while production of the ROI may be unaffected. We therefore hypothesized that SP-A inhibited ROI detection by scavenging ROI produced by the macrophage, inhibiting NADPH oxidase activity, or both.

The literature provides some evidence to support the possibility of SP-A as an oxidant scavenger. SP-A has been demonstrated to reduce peroxidation of lipids and protect cells from oxidant-induced death (38), and Kuzmenko et al.<sup>4</sup> have shown an inhibition of SP-A-mediated liposome aggregation after oxidation of SP-A, suggesting the protective mechanism includes oxidative modification and functional inactivation of SP-A. These modifications may include nitration, oxidation, and/or chlorination of tyrosine residues in SP-A by peroxynitrite (ONOO<sup>-</sup>) (39), nitrite (NO<sub>2</sub><sup>-</sup>), and HOCl, or NO<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> in the presence of myeloperoxidase (40). We examined the effects of 1–100  $\mu$ g/ml SP-A on superoxide generated by the reaction of xanthine and xanthine oxidase, and found no inhibition of superoxide as detected by CytC reduction. These results are in agreement with those of Katsura et al. (13), who found no inhibition of superoxide by 1–10  $\mu$ g/ml rat SP-A in a cell-free system. We also used the EPR spin trap DMPO as a more sensitive probe to study superoxide through the xanthine/xanthine oxidase reaction, but found no effect by up to 500  $\mu$ g/ml SP-A, which is 25- to 50-fold higher than the concentrations used in our cellular ROI assays. These data do not support scavenging of superoxide by SP-A.

We next examined the effects of SP-A on the activity of the NADPH oxidase. There are two general methods of studying NADPH oxidase activity: measuring cyanide-independent oxygen consumption, and measuring glucose consumption through the hexose monophosphate shunt (HMPS). The HMPS generates NADPH and pentoses from NADP<sup>+</sup> and glucose in a series of enzymatic reactions. In neutrophils, the primary consumer of NADPH is the NADPH oxidase; therefore, the activity of the HMPS has been indirectly linked to the activity of the NADPH oxidase (41). Although this assay has been used extensively to study HMPS activity in neutrophils, its use in macrophages has been rare. We began our investigation of the effects of SP-A on the NADPH oxidase by optimizing this assay for detection of HMPS activity in human macrophages in response to OpZy, but were unable to detect a significant inhibition of HMPS activity in the presence of SP-A (data not shown). Our assay used a single time point of 30 min, a time at which significant inhibition of ROI could be seen by both DCF and EPR spin trapping. However, glucose consumption through the HMPS in relation to NADPH oxidase activity has not been well studied in human macrophages. Therefore, the temporal kinetics of HMPS activity, the overall stores of preformed NADPH in the M $\phi$ , and the low activity of the M $\phi$  NADPH oxidase compared with that of neutrophils may all have

<sup>4</sup> Kuzmenko, A. I., H. Wu, J. P. Bridges, and F. X. McCormack. Surfactant protein oxidation: surfactant lipid peroxidation damages surfactant protein A and inhibits interactions with phospholipid vesicles. *Submitted for publication.*



been contributing factors to the lack of effects by SP-A in this assay system.

We therefore measured oxygen consumption as a direct, sensitive, and more proximal measurement of NADPH oxidase activity. There are two major mechanisms of respiration in the phagocyte: basal metabolism through mitochondrial respiration, and the respiratory burst through the NADPH oxidase. Macrophages have significant mitochondrial metabolic activity, even at rest. Therefore, we found it necessary to inhibit mitochondrial respiration through the addition of cyanide. We directly measured the  $pO_2$  in our macrophage culture system over time using a new oxygen-sensitive probe, LiNc-BuO (18). We found SP-A significantly inhibited the increase in macrophage oxygen consumption rate in response to both OpZy and PMA. Macrophage oxygen consumption in response to stimulation was due to NADPH oxidase activity, because treatment with DPI, an inhibitor of both mitochondrial and NADPH oxidase respiration (42), abrogated PMA-induced oxygen consumption, while KCN, which inhibits mitochondrial respiration, had no effect. These data support an inhibitory effect of SP-A on the activity of the macrophage NADPH oxidase. Therefore, we conclude that SP-A inhibits the production of ROI through the NADPH oxidase by human macrophages in response to stimuli by reducing the activity of the NADPH oxidase. To our knowledge, this is the first time that this probe has been used with macrophages. Our results demonstrate that it represents a powerful tool for assessing oxidative metabolism in cells that consume low levels of oxygen in response to stimuli, such as macrophages and nonprofessional phagocytes.

The phagocyte NADPH oxidase is a multiprotein complex consisting of both cytosolic and membrane-bound protein components. In the resting cell, the NADPH oxidase is inactive and unassembled. Ligation of macrophage phagocytic receptors initiates a signal cascade resulting in phosphorylation of p47<sup>phox</sup> in the cytosol, which causes translocation of p47<sup>phox</sup>, p67<sup>phox</sup>, and p40<sup>phox</sup> together from the cytosol to the membrane cytochrome b<sub>558</sub>, which is composed of gp91<sup>phox</sup> and p22<sup>phox</sup>. Together with GTP-binding proteins Rap1a and RAC2, these proteins form the catalytically active NADPH oxidase complex (2). Studies in neutrophils have shown that assembly of a functional NADPH oxidase requires the translocation of cytosolic NADPH oxidase component p47<sup>phox</sup> to the membrane component cytochrome b<sub>558</sub> (21, 22). To investigate the mechanism(s) by which SP-A inhibits NADPH oxidase activity, we therefore examined colocalization of p47<sup>phox</sup> with OpZy phagosomes using immunofluorescent staining and confocal microscopy. SP-A significantly reduced the number of p47<sup>phox</sup>-positive OpZy. Additionally, SP-A reduced the intensity of p47<sup>phox</sup> staining in those OpZy that were positive. This reduction in both the absolute number and relative magnitude of p47<sup>phox</sup>-positive OpZy phagosomes suggests that a mechanism of the reduction in NADPH oxidase activity by SP-A is decreased association of p47<sup>phox</sup> with the phagosomal membrane, which may in turn indicate a decreased number of functional NADPH oxidase complexes.

Still unresolved is the molecular mechanism(s) by which SP-A inhibits the NADPH oxidase. Phosphorylation of the oxidase component p47<sup>phox</sup> has been linked to both oxidase activation (43) and deactivation (44) events. SP-A has been shown to induce a transient rise in macrophage intracellular  $[Ca^{+2}]_i$ ,<sup>5</sup> a secondary signaling molecule that together with diacylglycerol activates several isoforms of protein kinase C. It is therefore possible that alteration

of oxidase activity by SP-A is due to altered phosphorylation of oxidase components and/or oxidase-regulating signal transduction proteins. In addition to its ability to signal the macrophage, SP-A may interact with the oxidase itself: the putative extracellular region of the membrane-bound oxidase component gp91<sup>phox</sup> contains several N-linked glycosylation sites (45). SP-A in the extracellular space or phagosomal compartment may bind gp91<sup>phox</sup> carbohydrates (N-acetyl-glucosamine and galactose (46)) via the carbohydrate recognition domain of SP-A, altering the conformation of gp91<sup>phox</sup> in the membrane and destabilizing the NADPH oxidase complex. The potential mechanisms of SP-A effects on NADPH oxidase assembly, activation, and/or disassembly are under investigation in our laboratory.

In summary, we have demonstrated that SP-A reduces the oxidative response of human macrophages to stimuli. This effect is independent of an effect of SP-A on phagocytosis. Our data do not support a role for SP-A in the scavenging of superoxide, but rather, they indicate SP-A inhibition of the respiratory burst is due to an effect of SP-A on the activity of the NADPH oxidase, which may be due at least in part to reduced association of p47<sup>phox</sup> with the phagosome. Although the production of ROI is an important mechanism of antimicrobial activity in the macrophage, regulation of the level of ROI produced through the NADPH oxidase in response to stimulation may be important in the lung to avoid damaging host tissues and compromising gas exchange. The current study supports the notion that the pulmonary surfactant constituent SP-A contributes to the alternate activation phenotype of alveolar macrophages and the maintenance of an anti-inflammatory environment in the normal, healthy lung.

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