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# Exercise Enhances Surfactant-Mediated Phagocytosis in Bronchoalveolar Macrophages

**Shu-Hui Su, Hsiun-ing Chen and Chauying J. Jen**

*Department of Physiology, College of Medicine*
*National Cheng Kung University*
*Tainan 701, Taiwan, R.O.C.*

## Abstract

**Severe exercise augments the phagocytic capability of bronchoalveolar macrophages (BAMs) in the absence of pulmonary surfactant, a lung immunity modulator *in vivo*. This study was to investigate whether the exercise effect on BAM phagocytosis is partially mediated by surfactant components. Male BALB/c mice (9-12 wk old) were divided into control and severe exercise groups. Mice in the exercise group received progressive treadmill running exercise until exhaustion. BAMs and lung lavage supernatant were collected under either sedentary or post-severe exercise conditions. Phagocytosis of IgG/C'-opsonized beads by BAMs was determined in the presence of lavage supernatant. Mannose, a monosaccharide competitor for the carbohydrate recognition domain of surfactant protein A (SP-A), and SP-A antibodies were applied to examine the role of SP-A in the exercise-induced facilitating effects on BAM phagocytosis. BAMs from either control or post-exercise animals had elevated phagocytosis of IgG/C'-opsonized beads when incubated with autologous lung lavage supernatant. The supernatant-mediated increase in BAM phagocytosis of IgG/C'-opsonized beads was dose-dependently inhibited by mannose or SP-A antibodies. In addition, higher concentrations of SP-A inhibitors were needed to inhibit BAM phagocytosis in post-exercise group than that in the control group. We also observed that SP-A inhibitors were ineffective in the absence of lung lavage supernatant. Furthermore, post-exercise, but not control, BAMs displayed time-dependent alterations in their membrane-bound SP-A amount during 30-min incubation with autologous lung lavage supernatant. SP-A plays a major role in the severe exercise-enhanced surfactant-mediated BAM phagocytosis.**

**Key Words: SP-A, opsonins, lavage supernatant, mannose, treadmill exercise, mice**

## Introduction

The respiratory system is relatively susceptible to infectious organisms. As the lung is frequently exposed to a wide range of potential allergens and microbial pathogens, bronchoalveolar macrophages (BAMs) plays a major and immediate defensive role in the recognition and clearance of such foreign materials (28). During the defense processes, BAM activities are affected by surrounding pulmonary surfactant that forms a cover layer on the apical side of alveolar epithelium. Pulmonary surfactant is composed of phospholipids (~80%), cholesterol (~10%), and proteins (~10%). Surfactant protein A (SP-A), the most abundant surfactant protein, binds a variety of organisms and particles (9). SP-A is structurally and functionally homologous to the serum complement C1q and mannose-binding proteins (14). They act as opsonins for leukocytes to catch the foreign particles. BAMs bind SP-A *in vitro* through specific surface receptors and appear to internalize the protein (13). In the presence of SP-A, the phagocytosis of opsonized particles and certain bacteria by BAMs is largely enhanced (24).

Epidemiological studies indicate that physical exercise exerts profound effects on the lung immunity, such as the incidence of upper respiratory tract infections (3, 17) and the prevalence and mortality

Corresponding author: Professor Chauying J. Jen, Department of Physiology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan, R.O.C. Tel: 886-6-235-3535 ext. 5439, Fax: 886-6-208-8521.
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rates for various site-specific cancers (1). In animal studies, treadmill exercise increases the NK-cell cytotoxic activity and decreases the lung retention of tumor cells (5, 11). However, even after the depletion of NK cells from the circulation, animals subjected to exercise still show lower retention of tumor cells (11). Therefore, it seems likely that BAMs, the major residential leukocytes in the lungs, may actively participate in the exercise-enhanced lung immunity. In fact, previous studies have demonstrated that preformed pulmonary surfactant is vigorously secreted from type II epithelial cells when the lung is exposed to certain stimuli, *e.g.*, hyperventilation (15) and physical exercise (16). These studies suggest that elevated surfactant turnover may be associated with physical exercise.

SP-A has been reported to enhance the uptake of IgG-, C1q-, or serum-coated particles by alveolar macrophages (24, 26). Our previous studies have shown that severe exercise in BALB/c mice enhances the phagocytosis of nonopsonized beads by surfactant-wash out BAMs (19, 20). Whether the surfactant plays any role in the effect of severe exercise on BAM phagocytosis remained to be answered. In the current study, we demonstrated that SP-A in pulmonary surfactant played an important role in exercise-enhanced BAM phagocytosis of IgG/complement-opsonized particles.

## Materials and Methods

*Reagents*

Carboxylate-modified fluorescent latex beads (2 μm in diameter), bovine serum albumin, and purified mouse IgG were purchased from Sigma (St. Louis, MO, USA). An affinity-purified goat anti-human SP-A polyclonal antibody (sc-7699) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human SP-A and SP-D sera were gifts from Dr. J-Y Wang, Dept. of Pediatrics, College of Medicine, National Cheng Kung University (25). Goat anti-rabbit IgG Alexa Fluor 488-conjugate and Alexa Fluor 546-conjugate were purchased from Molecular Probes (Eugene, OR, USA). Other chemical reagents used in this study were purchased from Merck (Darmstadt, Germany).

*Animals and Exercise Regime*

This study was conducted in conformity with the policies and procedures detailed in the *Guide for Animal Care and Use of Laboratory Animals*. Male BALB/c (H-$2^d$) mice (9-12 wk old) were purchased from the National Cheng Kung University Animal Center (Tainan, Taiwan). The mice were randomly divided into two groups, namely sedentary control and severe exercise. As described in our previous report (20), mice in the exercise group ran on a treadmill starting at 9 m/min for 3 min, followed by a 2 m/min increment every 3 min until a speed of 17 m/min was reached, and then continued to run with 1 m/min increment every 3 min until exhaustion. They were usually exhausted at 24 m/min with a total running time of about 36 min. The exercise-induced increase of circulating leukocytes was about 100%, in consistent with previous studies (19, 20). To avoid novel effects, the sedentary control mice were placed on the treadmill for 10 min without exercise.

*Isolation of BAMs*

Animals were anesthetized by intraperitoneal injection of 0.1-0.15 ml sodium pentobarbital (50 mg/ml). The murine lungs were filled and flushed with 1 ml of pre-warmed PBS (145 mM NaCl, 5 mM KCl, 9.35 mM $Na_2HPO_4$, 1.9 mM $KH_2PO_4$, 5.5 mM glucose, pH 7.4). In order to have enough BAMs for our experiments, this procedure was repeated to obtain a total volume of about 6 ml of bronchoalveolar lavage per animal. BAMs were collected by centrifugation at 800 × g for 8 min at 4°C and were resuspended in PBS for phagocytosis assay. The percentage of BAM in bronchoalveolar lavage was measured using Wright's differential leukocyte staining. BAMs were the major cell type (95%) collected in all murine bronchoalveolar lavage. The viability of cells was greater than 98% as revealed by trypan blue exclusion test. There was no difference in BAM count from the 6-ml lavage between control and exercise groups, *i.e.*, about $8\times10^5$ cells were collected from each mouse.

*Phagocytosis of Nonopsonized and Opsonized Latex Beads by BAMs*

The nonopsonized and opsonized beads were prepared as described in details previously (20). BAMs were incubated for 1 h with the bead suspension (cell-to-bead ratio = 1:30) in divalent cation-containing PBS or lavage supernatant (0.15 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.03 mM $MgSO_4$) at 37°C. Cells were subsequently washed with cold PBS to eliminate free particles, and thereafter fixed with 4% paraformaldehyde. The fluorescence histogram of macrophages was measured by a flow cytometer (FACSort, Becton Dickinson, San Jose, CA, USA). In our hands, each ingested bead contributed to about 100 fluorescence units. The fluorescence intensity below 50 units was regarded as no bead ingestion by BAMs. Results were presented as averaged number of ingested beads per cell (*i.e.*, phagocytosis index, PI).

To further assess the role of SP-A in the BAM phagocytic capacity, we treated BAMs with mannose, a monosaccharide competitor for the carbohydrate recognition domain of SP-A, or an antiserum against the whole SP-A protein in some experiments.

*Fluorescent Staining of SP-A in BAMs*

BAMs were isolated from bronchoalveolar lavage, and were used either immediately or after incubation with autologous lavage supernatant at 4°C for 10 min or 30 min. They were subsequently washed and fixed in 4% paraformaldehyde for 1 h. After fixative removal, the cells were incubated with antiserum against the whole SP-A protein. These cells were further labeled with fluorescence-conjugated secondary antibody. They were washed twice in PBS and analyzed by flow cytometry within 1 h or directly observed under a fluorescence microscope.

*Data Analysis*

Data were shown as mean ± SE, and *n* was the number of animals in each group. Results were analyzed by paired Student's *t*-test for lavage supernatant effects or by unpaired Student's *t*-test for exercise effects. The time-dependent binding of SP-A was analyzed by one-way ANOVA and Student-Newman-Keuls contrast procedures were further performed when significant effects were found. The statistics software SigmaStat was used for data analysis. The differences were considered to be significant when $P < 0.05$.

## Results

*Effects of Bronchoalveolar Lavage Supernatant on BAM Phagocytosis of IgG/C'-Opsonized Beads*

In our hands, the protein content in the lavage supernatant decreased drastically with repeated flush, and the first ml of lung lavage contained about 70% of cumulative protein amount (data not shown). Only the fresh supernatant from the first 1 ml of lung lavage was used in our experiments. In control or post-exercise animals, BAM phagocytosis of IgG/C'-opsonized beads was elevated when autologous supernatant was present (Fig. 1). Moreover, the BAM phagocytosis index in exercise group was significantly greater than that in the control. The supernatant-mediated increases in BAM phagocytosis index of opsonized beads, indicated by the ratio of differences between supernatant and saline treatments over the saline treatment, were measured in each individual animal and then averaged as 29 ± 6% and 54 ± 9% in the control (n = 5) and exercise (*n* = 6) groups, respectively ($P < 0.05$).

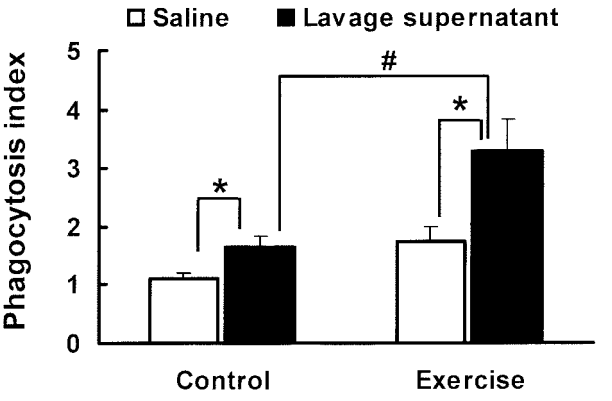


Fig. 1. Effects of autologous lavage supernatant on BAM phagocytosis of IgG/C'-opsonized beads. BAMs were incubated with either saline or lavage supernatant freshly prepared from the same animal ($n = 5$). Data were analyzed by either paired *t*-test (supernatant *vs.* saline) or unpaired *t*-test (exercise *vs.* control). * $P < 0.05$, supernatant *vs.* saline; # $P < 0.05$, exercise *vs.* control.

In a previous study, we have reported that severe exercise enhances the phagocytosis of nonopsonized beads by BAMs in the absence of lavage supernatant (19, 20). The current study showed that bronchoalveolar lavage supernatant from post-exercise animals enhanced the control BAM phagocytosis of nonopsonized beads (26 ± 7% increase, $p = 0.001$, $n = 10$), whereas the supernatant from control animals was ineffective. In contrast, BAMs from post-exercise animals showed elevated phagocytic capacity of nonopsonized beads, but their activity was not increased further by the addition of supernatant from either post-exercise or control animals (data not shown).

*Inhibitory Effects of Mannose and SP-A Antibody Preparations on Exercise- or Supernatant-Enhanced BAM Phagocytic Capacity*

Since the basal values of BAM phagocytosis index were different between control and exercise groups, data were presented as the percent difference of phagocytosis index from the corresponding vehicle-treated BAMs. Our results showed that the enhanced effect of bronchoalveolar lavage supernatant on BAM phagocytosis of opsonized beads was dose-dependently inhibited by mannose (Fig. 2, upper panel). Higher amounts of mannose were needed for maximal inhibition in the exercise group than in the control. Consistent with the fact that mannose at millimolar concentration range is pro-phagocytic (6), high doses of mannose, *i.e.*, 1-10 mM, showed a slightly reduced inhibitory effect on BAM phagocytosis. D-mannitol, a stereoisomer of mannose,

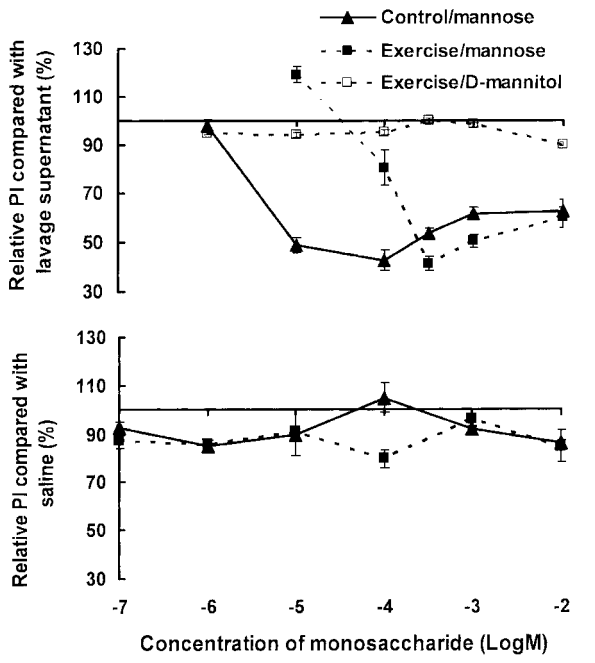


Fig. 2. Effects of mannose on BAM phagocytosis of IgG/C'-opsonized beads. BAMs were treated with different concentrations of mannose during phagocytosis in the presence (upper panel) or absence (lower panel) of autologous lavage supernatant. Data were calculated by the following formula: $[(PI_{monosaccharide} - PI_{vehicle}) / PI_{vehicle}] \times 100\%$, where PI (phagocytosis index) is defined as the averaged number of ingested beads per cell and vehicle is lavage supernatant (upper panel) or saline (lower panel). ($n$ = 4)

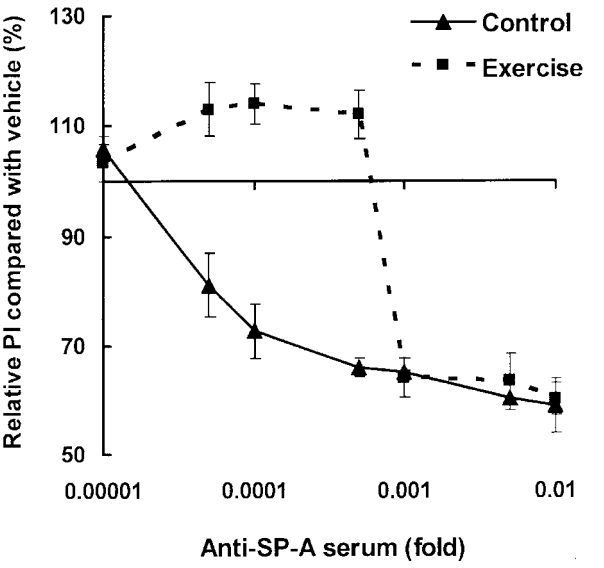


Fig. 3. Effects of SP-A antiserum on supernatant-mediated BAM phagocytosis of IgG/C'-opsonized beads. BAMs were incubated with different dilutions of antiserum that recognized the whole SP-A protein. Data were calculated by the following formula: $[(PI_{antiserum} - PI_{vehicle}) / PI_{vehicle}] \times 100\%$, where vehicle is lavage supernatant. ($n$ = 4).

was ineffective in blocking the supernatant-mediated BAM phagocytosis of opsonized beads. Maltose, another monosaccharide competitor with weak affinity for SP-A and high affinity for SP-D, partially inhibited the supernatant-mediated BAM phagocytosis. However, severe exercise did not significantly affect the inhibitory effects of maltose (data not shown). As a negative control, the inhibitory effect of mannose was minimal when BAM phagocytosis was performed in the absence of supernatant (Fig. 2, lower panel).

The hypothesis that SP-A was the major effective component in the bronchoalveolar lavage was further supported by experiments using an antiserum against the whole SP-A protein (Fig. 3). SP-A antibodies dose-dependently blocked the supernatant-enhanced BAM phagocytosis. Besides, more concentrated SP-A antibodies were needed to block the supernatant-enhanced BAM phagocytosis in the exercise group. The rabbit normal serum showed minor pro-phagocytic effect, whereas anti-SP-A was totally ineffective in the absence of lavage supernatant (data not shown). The polyclonal antibody against SP-A carboxyl terminus was also effective in either control or post-exercise conditions, *i.e.*, up to 25% or 35% inhibition, respectively. Moreover, the inhibitory effects of these two SP-A antibody preparations on supernatant-mediated phagocytosis were additive (Fig. 4). The lavage supernatant-mediated enhancement of phagocytosis in the exercise group was completely abolished by a combined treatment of antiserum and SP-A polyclonal antibody. Neither the polyclonal antibody nor the antiserum alone affected BAM phagocytosis in the absence of supernatant. In contrast, the anti-SP-D serum was much less effective in blocking the lavage supernatant-mediated phagocytosis by BAMs from post-exercise animals (less than 8 % inhibition at maximal strength).

*Effect of Severe Exercise on the Binding of SP-A to BAMs*

Assuming that the binding of SP-A to BAMs was involved in the process of supernatant-mediated BAM phagocytosis, we measured the relative binding amount of SP-A in control and post-exercise BAMs immediately after its isolation from the lung lavage. The amount of BAM-associated SP-A, as indicated by the fluorescence intensity associated with labeled SP-A antibody bound on the BAM surface, was the same in two groups (exercise 41.9 ± 2.8 *vs.* control 41.3 ± 5.0, $n$ = 6). Although antiserum recognizes various exposed epitopes of BAM-bound SP-A, in principle only a fraction of them can serve as an active opsonin in facilitating BAM phagocytosis. Moreover, the SP-A-mediated BAM phagocytosis should correlate with the kinetics of SP-A binding on and

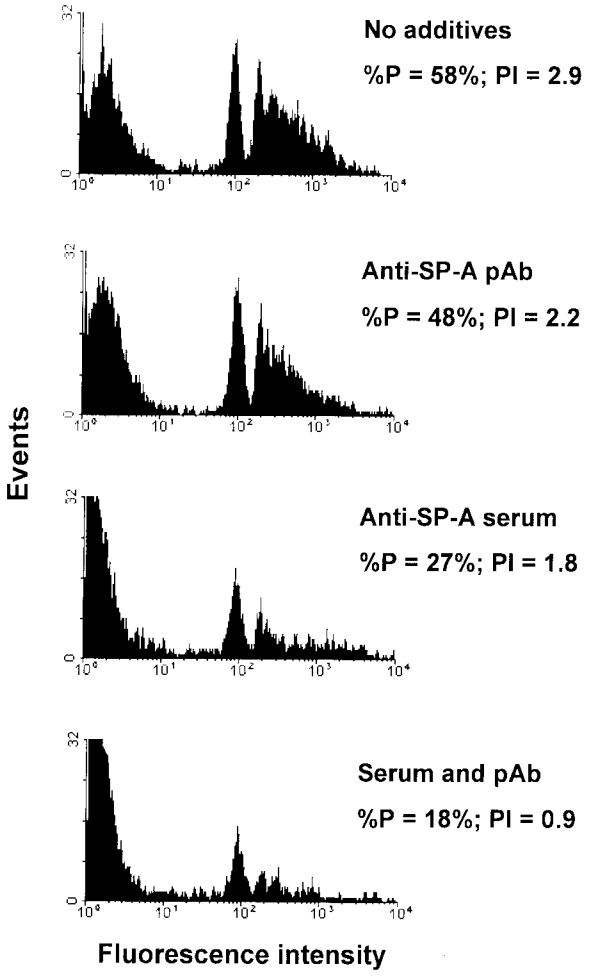


Fig. 4. Suppression of BAM phagocytosis of IgG/C'-opsonized beads in the presence of lavage supernatant by antiserum and polyclonal antibody against SP-A. Flow cytometry data showed that IgG/C'-opsonized fluorescently labeled latex beads were ingested by BAMs from exercised animals. The fluorescence intensity below 50 units was regarded as no bead ingestion by BAMs. Percentage of phagocytic cells (%P, the number of cells that ingested at least one bead divided by the total cell number) and phagocytosis index (PI, the total ingested beads divided by the total cell number) were calculated and shown next to the respective histogram.

internalization by BAMs. Therefore, we further examined the time-dependent changes of the BAM membrane-bound SP-A during the incubation period with the autologous supernatant. The results demonstrated that the SP-A binding underwent dynamic changes in post-exercise BAMs, but not in the controls (Fig. 5).

## Discussion

Although physical exercise has been reported to affect the secretion of surfactant and the function of immune cells in the lungs, how they work together

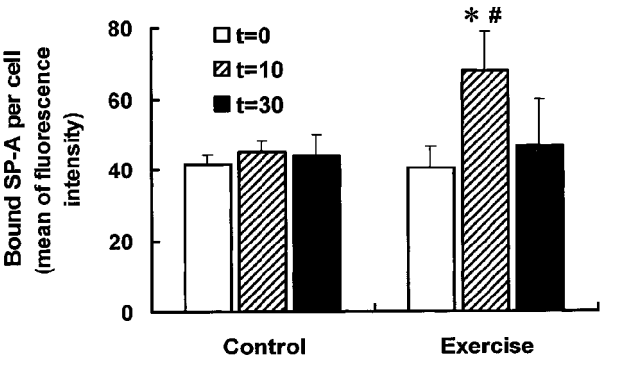


Fig. 5. The BAM-associated SP-A after incubation with autologous supernatant for various time periods. After BAMs were fixed at different incubation times with lavage supernatant, their surface-associated SP-A was immunostained with anti-SP-A serum/fluorescence-conjugated secondary antibody. Data are presented as mean fluorescence intensity and analyzed by one-way ANOVA ($n = 3$). *$P < 0.05$ ($t = 10$ min *vs.* t = 0 min or t = 30 min in the same group); #$P < 0.05$ (exercise *vs*. control).

remains unclear. Our previous report has shown that severe exercise enhances BAM phagocytosis of nonopsonized beads in the absence of surfactant (20). In this study, the functional performance of BAMs isolated from sedentary and post-exercise mice were examined in the presence of autologous surfactant-containing lung lavage supernatant. Results demonstrated that BAMs ingested more IgG/C'-opsonized beads in the presence of supernatant, and that this phagocytic activity was further increased by severe exercise. Moreover, the lavage supernatant-enhanced BAM phagocytosis of opsonized beads was largely inhibited by SP-A blockers, *i.e*., mannose and specific antibodies. Taken together, the BAM phagocytic activity in post-exercise animals could be modulated *via* both surfactant-dependent and surfactant-independent mechanisms.

To our knowledge, this study is the first to assess the role of surfactant in BAM phagocytosis using the original lavage surfactant that was freshly isolated. Addressing the short-term effects of acute exercise on either surfactant or BAMs became possible because the first collected autologous lavage supernatant was used with minimal handling time. On the contrary, previous studies usually used concentrated surfactant either prepared by centrifugation of the pooled lavage supernatant from numerous animals or from patients suffering alveolar proteinosis (22, 24, 26). Possibly due to the diluting effect of lung lavage, we were unable to quantify SP-A amount in the supernatant. Nonetheless, the treatment of SP-A blockade abolished the exercise effect, implying that severe exercise might increase surfactant secretion. This hypothesis needs to be further studied.

Our results support the notion that pulmonary surfactant collected after a severe exercise session would either trigger the activation of BAMs for ingesting nonopsonized beads or serve as the opsonin for BAM phagocytosis of opsonized beads. To test whether the BAM phagocytosis activity of nonopsonized beads was influenced by surfactant or not, we examined four different combinations of BAMs and lavage supernatant, *i.e.*, post-exercise BAMs with post-exercise or control supernatant, and control BAMs with post-exercise or control supernatant. Only the supernatant from the post-exercise mice enhanced the phagocytosis of nonopsonized beads by control BAMs. Moreover, severe exercise enhanced the autologous supernatant-mediated increase in BAM phagocytosis of opsonized beads (Fig. 1). The mechanical stretch during severe exercise-induced hyperpnea may directly enhance the surfactant secretion, as elevated ventilation rate with accompanying lung distension and altered intracellular pH in type II epithelial cells stimulates surfactant secretion (4). Generally speaking, severe exercise elevates plasma levels of stress hormones. In animal studies, all four surfactant proteins are increased as a result of elevated plasma levels of the stress hormone corticosterone (2). Previous reports have demonstrated that macrophages pre-incubated with concentrated surfactant significantly enhance their phagocytosis of opsonized bacteria and erythrocytes (24). Thus, the exercise-enhanced effects on surfactant-mediated BAM phagocytosis may be triggered either by physical factors or by hormonal factors.

Existing evidence indicates that pulmonary surfactant components, especially the surfactant-associated protein SP-A and SP-D, affect the BAM function, including phagocytosis, chemotaxis, and secretion (7, 27). They bind to specific carbohydrates on the surface of bacteria and viral pathogens and act as an opsonin to mediate immunological responses. We propose that SP-A was a mediator in lavage supernatant-enhanced BAM phagocytosis of opsonized beads in either resting or post-exercise conditions for the following reasons: First, our results showed that the lavage supernatant-mediated phagocytosis of IgG/C'-opsonized beads by either control or post-exercise BAMs was inhibited by mannose or anti-SP-A serum (but not by anti-SP-D serum) in a dose-dependent manner (Figs. 2 and 3). Second, more mannose or anti-SP-A serum was needed to inhibit opsonized phagocytosis in the exercise group than in the control group. Third, as the recycle of cell-associated SP-A was more pronounced under post-exercise conditions (Fig. 5), there should be more SP-A receptors on the surface of post-exercise BAMs. Therefore, it is feasible that severe exercise enhances the interactions between SP-A and BAMs in the lung lavage.

Although SP-A is the major protein in pulmonary surfactant, it does not appear to have an essential role in reduction of surface tension, since SP-A deficient mice survive and have normal lung compliance and volume (10). In contrast, these SP-A deficient mice are more susceptible to infection with group B *Streptococcus*, *Pseudomonas aeruginosa*, respiratory syncytial virus, and *Mycoplasma pulmonis* (8). Several studies have reported that purified human SP-A stimulates the macrophage uptake of certain gram-positive bacteria such as *Staphylococcus aureus and S. pneumoniae* (24), gram-negative bacteria such as *Esherichia coli* (18), and *Haemophilus influenzae* type A (22). It is possible that severe exercise also enhances BAM phagocytosis of these microbes *in vivo*.

As SP-A at physiologically relevant concentrations stimulates directional actin polymerization and chemotaxis in BAMs (23), it is likely that the exercise-induced surfactant secretion facilitates SP-A-mediated particle internalization by BAMs. The association of SP-A with BAMs would be an essential step for SP-A acting either as an opsonin or as an activation ligand. After pre-incubation of BAMs with autologous lavage supernatant for different time periods, a dynamic alteration of surface SP-A was observed in BAMs from post-exercise mice (Fig. 5). Two types of transmembrane proteins, C1qRp and SPR 210, have been identified as SP-A receptors expressed on alveolar macrophages and type II epithelial cells (21). Both receptors participate in SP-A-mediated phagocytosis and involve in the turnover of SP-A. Our results showed that the inhibitory effects of anti-SP-A polyclonal antibody and anti-SP-A antiserum on surfactant-mediated phagocytosis of opsonized beads by post-exercise BAMs were additive, *i.e.*, the maximal inhibition from the treatment of antibody or antiserum alone was about 25% or 40% reduction, while a combination treatment yielded approximately 70% reduction (Fig. 4). Moreover, the binding of SP-A antiserum and polyclonal antibody in terms of immunostaining intensity on BAMs were also additive (data not shown). Since SP-A is capable of interacting with cell surface receptors *via* either its collagen-like domain or its carboxyl terminus (12, 13), the additive effects on SP-A binding or the phagocytosis inhibition exerted by two different antibody preparations were expected.

While the major surfactant component, *i.e.*, various lipids, shows suppressive effects on the BAM function, SP-A, a minor surfactant component, appears to have opposite effects (27). This study firstly demonstrates that severe exercise facilitates lavage

supernatant-mediated BAM phagocytosis of opsonized particles, and that SP-A plays an important role in this process. An enhanced SP-A-mediated BAM phagocytosis would be protective against foreign particles inhaled during severe exercise.

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