

Transport of AOPP-Albumin into Human Alveolar Epithelial A549 Cells

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ABSTRACT - Purpose. Alveolar clearance of proteins, such as albumin, plays an essential role in recovery from lung injuries. Albumin is known to be oxidized by reactive oxygen species (ROS), leading to generation of advanced oxidation protein products (AOPP)-albumin in the alveolar lining fluid. In this study, we aimed to characterize the uptake of FITC-labeled AOPP-albumin (FITC-AOPP-albumin) into human alveolar epithelial cell line, A549. **Methods.** FITC-AOPP-albumin uptake into A549 cells and its effect of ROS generation was evaluated using fluorescence spectrometer and flow cytometry, respectively. **Results.** FITC-AOPP-albumin was taken up by A549 cells in a time- and temperature-dependent fashion, and showed saturation kinetics with a K_m value of 0.37 mg/mL. The uptake of FITC-AOPP-albumin was suppressed by phenylarsine oxide, a clathrin-mediated endocytosis inhibitor, but not by indomethacin and nystatin, caveolae-mediated endocytosis inhibitors, or 5-(N-ethyl-N-isopropyl) amiloride, a macropinocytosis inhibitor. AOPP-albumin induced ROS generation in A549 cells, suggesting that alveolar clearance of AOPP-albumin should be important to prevent further ROS generation. **Conclusion.** AOPP-albumin is transported into alveolar epithelial cells through clathrin-mediated endocytosis, which may be important to prevent further ROS generation.

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INTRODUCTION

The surface of alveolar region is composed of two main cell types: the alveolar type I and type II cells. Type I epithelial cells covering approximately 90–95% of the alveolar surface area are squamous cells, and play a fundamental role in gas change (1). Type II epithelial cells are cuboidal in shape and occupy 5–10% of the surface area (1). Additionally, type II epithelial cells have secretory organelles called lamellar bodies that are crucial for surfactant production and secretion (2).

There are various proteins including albumin, immunoglobulin G (IgG), IgA, and transferrin in alveolar lining fluid (1, 3, 4). In general, the albumin concentration under physiological condition is 5 mg/mL, which is much lower than its concentration in plasma (40 mg/mL). However, the concentration of albumin in the alveolar fluid increases to 75–95% of the levels in plasma during pulmonary edema. Large quantities of insoluble proteins are observed in air spaces of patients with acute respiratory distress syndrome (ARDS) (5). Therefore, alveolar clearance of proteins, such as

albumin, is critical to the process of recovery from pulmonary edema.

In response to inflammatory stimuli in lung, lung cells, such as alveolar epithelial cells and activated macrophages, produce reactive oxygen species (ROS), which oxidize lung proteins and inhibit their function. Increasing levels of ROS decrease the levels of enzymes in the antioxidant defense system, including superoxide dismutase and catalase, as well as scavengers such as glutathione, and vitamin E and C (6, 7). During ARDS, inflammatory mediators such as cytokines, chemokines, and adhesion molecules indirectly mediate ROS production and lead to further damage (8). Albumin, an abundant and important circulating antioxidant, is oxidized by ROS, producing advanced oxidation protein products (AOPP)-albumin (9). This AOPP-albumin

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production may occur when ROS are generated during lung injury, so alveolar clearance of AOPP-albumin may be important for recovery from pulmonary edema.

Clearance mechanisms for various peptides and proteins from the alveolar space have been widely investigated and transcytosis is suggested to be the primary mechanism of protein and peptide absorption across alveolar epithelial cells (3, 4). In addition, we have reported that albumin was taken up by endocytosis in human and rat alveolar epithelial cell lines, A549 and RLE-6TN, in addition to rat primary cultured alveolar epithelial cells (10-13). The uptake of albumin in these cells was inhibited by chlorpromazine and phenylarsine oxide, inhibitors of clathrin-mediated endocytosis. Inhibitors of caveolae-mediated endocytosis, such as indomethacin, methyl- β -cyclodextrin, and nystatin did not suppress the uptake of albumin into these cells. Furthermore, knockdown of clathrin heavy chain by siRNA in RLE-6TN cells significantly decreased its mRNA expression and cellular uptake of albumin, indicating that albumin is endocytosed into alveolar epithelial cells through the clathrin-mediated pathway, but not the caveolae-mediated pathway (14, 15). However, the transport of AOPP-albumin into alveolar epithelial cells is not well understood.

In this study, we prepared FITC-labeled AOPP-albumin (FITC-AOPP-albumin) from native albumin and investigated the mechanism underlying transport of AOPP-albumin into A549 cells. In addition, the effect of albumin and AOPP-albumin on ROS production was examined to address the role of albumin in the alveolar space and the importance of alveolar clearance of AOPP-albumin.

MATERIALS and METHODS

Materials

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from MP Biomedicals (Solon, OH, USA). Trypsin-EDTA and penicillin-streptomycin were purchased from Life Technologies (Carlsbad, CA, USA). 5-(N-ethyl-N-isopropyl) amiloride (EIPA), indomethacin, nystatin and phenylarsine oxide (PAO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium azide (NaN_3) was purchased from Katayama Chemical Industries Co., Ltd (Osaka, Japan). 2-deoxy-D-glucose (2-DOG) was purchased from Kanto Chemical Co. (Tokyo,

Japan). All chemicals used in the experiments were of the highest purity commercially available.

Cell culture

A549 cells were cultured in DMEM containing 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% FBS in 5% CO_2 at 37°C, and subcultured every 7 days using 1 mM EDTA and 0.25% trypsin. The medium was replaced every 2 or 3 days.

Preparation of AOPP-albumin and fluorescein isothiocyanate (FITC)-labeled AOPP-albumin

BSA (20 mg/mL; fatty acid-free) and chloramine T (100 mM) were dissolved in phosphate-buffer (62.8 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 3.92 mM KH_2PO_4 ; pH 8.0) by incubating for 1 h at 37°C with bubbling 95% O_2 . Dialysis was performed for 24 h at 4°C, followed by lyophilization to obtain dried powdered AOPP-albumin.

The AOPP-albumin was dissolved in 0.1 M Na_2CO_3 - NaHCO_3 solution (20 mg/mL) and mixed with 15 mM FITC solution (1:2.5 AOPP-albumin:FITC). The mixture was incubated with shaking for 4 h at room temperature. The fraction with a fluorescence intensity of 500 nm (ex)/520 nm (em) was collected and dialyzed using a cellulose membrane for 24 h at 4°C for removal of free FITC. The resulting lyophilized powder was used as FITC-AOPP-albumin.

Uptake studies

A549 cells were seeded into 12-well plates at a density of 10×10^4 cells per well, and grown for 6 days. After the culture medium was removed, each well was washed twice and preincubated with phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.5 mM MgCl_2 and 0.1 mM CaCl_2 ; pH 7.4) containing 5 mM D-glucose (PBS-G) at 37°C for 10 min. PBS-G buffer containing FITC-AOPP-albumin (20 mg/mL) was then put into each well and the cells were incubated at 37°C or 4°C for 30, 60, 90, and 120 min.

For the inhibition studies, A549 cells were preincubated for 10 min with or without inhibitors as follows: PBS, PBS-G, 5 mM NaN_3 plus 2.5 mM 2-deoxy-D-glucose in PBS, PAO (5-20 μM) in PBS-G, 300 μM indomethacin in PBS-G, 50 μM nystatin in PBS-G, or 50 μM EIPA in PBS-G buffer. After the preincubation buffer was removed, the cells were incubated with 0.5 mL PBS-G buffer containing FITC-albumin with or without the inhibitors.

After the incubation buffer was aspirated, the cells were washed three times with 1 mL of ice-cold PBS. The cells were collected into 0.5 mL ice-cold PBS with a rubber policeman and the wells were rinsed with 0.5 mL ice-cold PBS for improving recovery of cells. Subsequent washes of the cells were performed by centrifugation at 9838 x g for 3 min at 4°C two times. After removal of the supernatant, the pellet was solubilized in 0.8 mL PBS containing 0.1% Triton X-100 without CaCl₂ and MgCl₂ at room temperature for 30 min, then centrifugation at 5600 x g for 3 min. Fluorescent analysis at 500 nm (ex)/520 nm (em) and protein assay (Lowry method) were performed using the supernatant.

Real-time PCR analysis

Total RNA of A549 cells was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics, Laval, QC). Reverse transcription of the total RNA into cDNA was performed using ReverTra Ace (TOYOBO, Osaka, Japan). Quantitative evaluation for indicated mRNA expression was performed by real-time PCR analysis using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the KAPA SYBR® FAST qPCR Kit (NIPPON Genetics, Tokyo, Japan), as previously described (16). The primer sequences were as follows: *CD36* sense 5'-GCCTCTCCAGTTGAAAACCC-3', antisense 5'-ACACAGGTCTCCCTTCTTTGC-3'; *SR-B1* (*SCRAB1*) sense 5'-GATCCCTATCCCCTTCTATCTCTCC-3', antisense 5'-CTTTTGTGCCTGAACTCCCTGTA-3'; human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) sense 5'-ACGGAAGCTTGTCATCAAT-3', antisense 5'-TGGACTCCACGACTACTCA-3'. The mRNA expression level of *GAPDH*, a housekeeping gene, was used for normalization for that of each gene.

Transfection with siRNAs and uptake studies of FITC-AOPP-albumin

A549 cells were seeded into 12-well plates at a density of 10 × 10⁴ cells per well. After 24 h, siRNAs specific for *CD36* and *SR-B1* (20 pmol/well) were transfected into the cells using Lipofectamine® 2000 (Thermo Fisher Scientific Inc., Massachusetts, USA), referring to the manufacturer's instructions. MISSION® siRNA Universal Negative Control (Sigma) was used as a

control in an identical manner. After 72 h, the efficiency of siRNA silencing was evaluated using the uptake study and real-time PCR analysis as described in sections of Uptake studies and Real-time PCR analysis, respectively.

ROS detection by flow cytometry

A549 cells were seeded into 12-well plates at a density of 10 × 10⁴ cells per well. After 24 h, the cells were treated with 1 mM H₂O₂, 5 mg/mL albumin, or 5 mg/mL AOPP-albumin for 24 h. In a second set of conditions, cells were treated with 0.5 mM H₂O₂ for 24 h, followed by incubation with 10 mM N-acetylcysteine (NAC) or 40 mg/mL albumin for 2.5 h. Flow cytometry analysis was performed as previously described to detect ROS by measuring the fluorescence of 7'-dichlorofluorescein (DCF) (17).

STATISTICAL ANALYSIS

Mean ± standard error of the mean (SEM) was given to each data. Statistically significant differences were determined by one-way ANOVA, followed by Tukey's test. Statistically significance was considerable when a *p*-value less than 0.05.

RESULTS

General characteristics of FITC-AOPP-albumin uptake into A549 cells

To understand the general characteristics of AOPP-albumin uptake into A549 cells, the time- and temperature-dependence of FITC-AOPP-albumin (5 mg/mL) uptake were examined. FITC-AOPP-albumin uptake increased linearly up to 90 min at 37°C and the uptake was much lower at 4°C at all time points (Figure 1A). In addition, the uptake of FITC-AOPP-albumin in A549 cells was saturable and was mediated by a single transport system as evidenced by the linear Eadie-Hofstee plot (Figure 1B). The *V*_{max} (maximum uptake rate) and *K*_m (Michaelis constant) values were 1.4 µg/mg protein/60 min and 0.37 mg/mL, respectively.

Effect of unlabeled AOPP-albumin and metabolic inhibitors on FITC-AOPP-albumin uptake into A549 cells

FITC-AOPP-albumin (5 mg/mL) uptake into A549 cells was significantly inhibited by the co-treatment of the cells with unlabeled AOPP-albumin (20 mg/mL) (Figure 2), indicating that a specific

transport system is associated with the uptake into A549 cells. In addition, pretreatment with metabolic inhibitors, NaN_3 plus 2-DOG,

significantly decreased FITC-AOPP-albumin uptake to 69% of the control uptake (Figure 2).

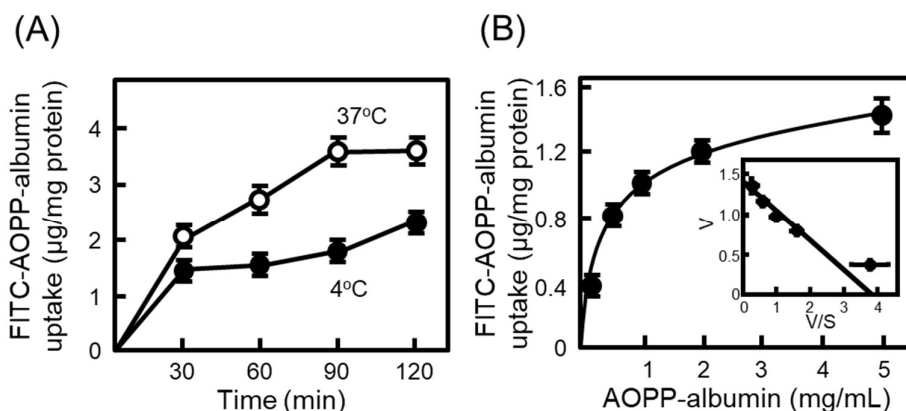


Figure 1. General characteristics of FITC- AOPP-albumin uptake into A549 cells. (A) The cells were incubated with FITC-AOPP-albumin (5 mg/mL) for 30, 60, 90, and 120 min at 37°C (open circles) or 4°C (close circles). (B) The cells were incubated with FITC-AOPP-albumin (0.1, 0.5, 1, 2, 5 mg/mL) for 60 min at 37°C or 4°C. The plot represents the specific uptake, found by subtracting the uptake at 4°C from the uptake at 37°C. The inset shows an Eadie-Hofstee plot. Each point represents the mean \pm SEM (n = 3).

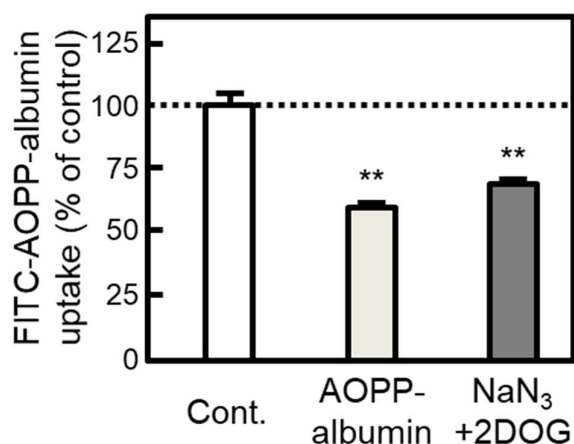


Figure 2. Effect of unlabeled AOPP-albumin and metabolic inhibitors on FITC-AOPP-albumin uptake into A549 cells. The cells were incubated with FITC-AOPP-albumin (5 mg/mL) in the absence or presence of unlabeled AOPP-albumin (20 mg/mL) or NaN_3 (5 mM) and 2-deoxy-D-glucose (2-DOG) (2.5 mM). Each value represents the mean \pm SEM (n = 3). Significant differences from the values found in the control cells are indicated as $**p < 0.01$.

Role of scavenger receptors in FITC-AOPP-albumin uptake into A549 cells

As several reports have demonstrated that AOPP-albumin is one of the ligands for CD-36 and

SR-B1 which are members of scavenger receptor class B, we focused on the role of these receptors in FITC-AOPP-albumin uptake into A549 cells. Transfection of A549 cells with siRNA specific for CD-36 and SR-B1 induced a significant decrease in the mRNA expression of CD-36 and SR-B1 to 31% and 22%, respectively, as compared to transfection with control siRNA (Figure 3A). Using these CD36 and SR-B1 knockdown cells, the uptake of FITC-AOPP-albumin (5 mg/mL) was examined. We found that the siRNA-mediated knockdown of CD36 and SR-B1 had no effect on FITC-AOPP-albumin uptake into A549 cells (Figure 3B).

FITC-AOPP-albumin uptake into A549 cells via endocytic pathway

We examined the possible endocytic pathway involved in FITC-AOPP-albumin uptake into A549 cells using various endocytosis inhibitors. PAO, an inhibitor of clathrin-mediated endocytosis, induced a significant decrease in FITC-AOPP-albumin uptake, in a concentration-dependent manner (Figure 4A). On the other hand, FITC-AOPP-albumin uptake was not affected by nystatin and indomethacin, which are inhibitors of caveolae-mediated endocytosis, or by EIPA, a macropinocytosis inhibitor (Figure 4B).

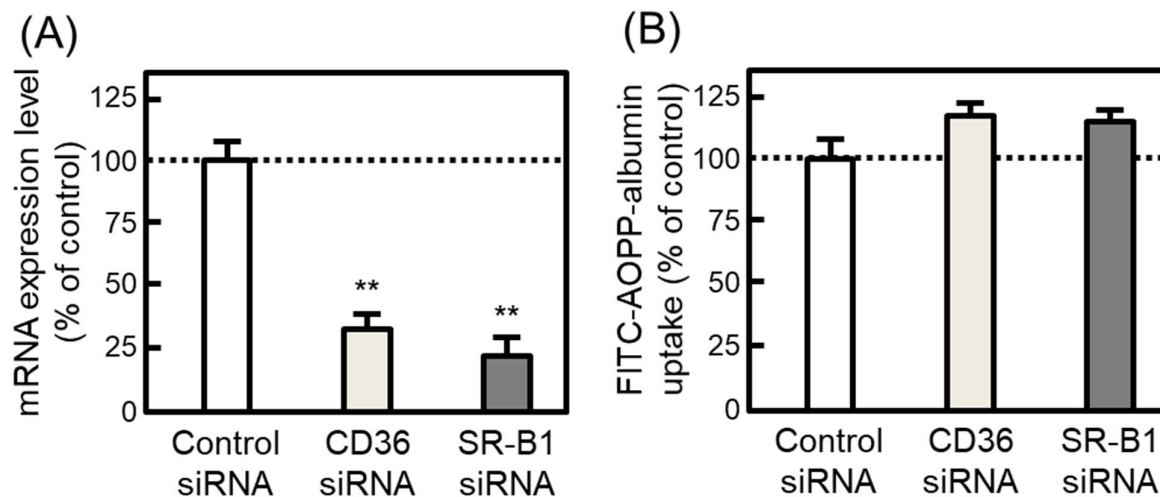


Figure 3. Role of scavenger receptors in FITC-AOPP-albumin uptake into A549 cells. MISSION[®] negative control, CD36-specific, or SR-B1-specific siRNA were transfected into A549 cells using Lipofectamine[®] 2000. (A) Real-time PCR analysis was performed 72 h after the transfection. (B) The cells were incubated with FITC-AOPP-albumin (5 mg/mL) for 60 min at 37°C. Each value represents the mean \pm SEM (n = 3). Significant differences from the values found in the control cells are indicated as ****** $p < 0.01$.

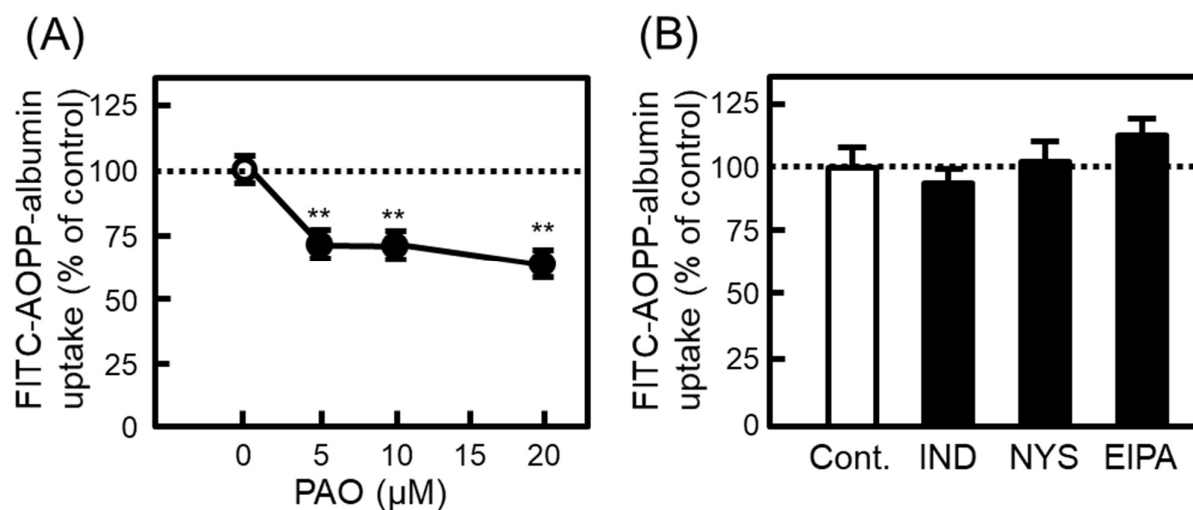


Figure 4. Endocytic pathway of FITC-AOPP-albumin uptake into A549 cells. (A) The cells were incubated with FITC-AOPP-albumin in the absence or presence of various concentrations of phenylarsine oxide (PAO) (5, 10, 20 μ M) for 60 min at 37°C. (B) The cells were incubated with FITC-AOPP-albumin in the absence or presence of indomethacin (IND) (300 μ M), nystatin (NYS) (50 μ M), or 5-(N-ethyl-N-isopropyl) amiloride (EIPA) (50 μ M). Each value represents the mean \pm SEM (n = 3). Significant differences from the values found in the control cells are indicated as ****** $p < 0.01$.

Effect of albumin and AOPP-albumin on ROS generation in A549 cells

Considering that ROS production is associated with lung injuries, such as pulmonary edema, it is

important to recognize the regulation of ROS generation by albumin and AOPP-albumin. As such, the effect of AOPP-albumin and albumin on ROS generation in A549 cells was examined. As shown

in Figure 5A, treatment of A549 cells with H₂O₂ (1 mM), a ROS inducer, or AOPP-albumin (5 mg/mL) for 24 h significantly increased the intracellular level of ROS, while albumin did not affect the level of ROS. The effect of albumin on H₂O₂-induced enhancement of ROS generation in A549 cells was also examined by treatment of A549 cells with H₂O₂ (0.5 mM) for 24 h, followed by incubation with albumin (40 mg/mL) or NAC (10 mM), a ROS scavenger, for 2.5 h. Both albumin and NAC significantly suppressed the elevation in ROS levels induced by H₂O₂ in A549 cells (Figure 5B).

DISCUSSION

It is well established that alveolar fluid clearance is markedly impaired in most patients suffering from ARDS and this impairment is associated with worse outcomes (18). In addition, the excess alveolar fluid clearance is an active metabolic process concerning vectorial transport of Na⁺ with water out of alveolar air space, following the osmotic gradient (19). Na⁺ transport from the apical surface to alveolar epithelial cells is associated with amiloride-sensitive Na⁺ channels (ENaC) and in part, by other, less well-characterized cationic channels (20, 21). Thus, the mechanism underlying alveolar fluid clearance mediated by Na⁺ transport is becoming clearer. Although clearance of various

proteins from the alveolar space is important for the recovery from pulmonary edema, limited information is available on the mechanisms, especially for proteins other than albumin (15, 22). In this study, the transport of AOPP-albumin, an oxidized albumin product, into A549 cells was investigated.

FITC-labeled AOPP-albumin was taken up by A549 cells in a time- and temperature-dependent manner and the uptake showed saturation kinetics, with V_{max} and K_m values of 1.3 μg/mg protein/60 min and 0.3 mg/mL, respectively. Our previous report demonstrated that albumin uptake was mediated by two types of transport systems in A549 cells, low and high affinity transport systems with K_m values of 5 mg/mL and 0.15 mg/mL, respectively (10). In this study, AOPP-albumin uptake by a single transport system was suggested by an Eadie-Hofstee plot, indicating that a high affinity transport system for albumin uptake may be involved in AOPP-albumin uptake into A549 cells. Anraku et al. have reported that chloramine-T induced oxidation of not only a cysteine residue (Cys34), but also of methionine residues at positions 298, 329 and 446 in subdomain IIA of albumin (23). These changes may suppress the low affinity transport system-mediated AOPP-albumin uptake in A549 cells.

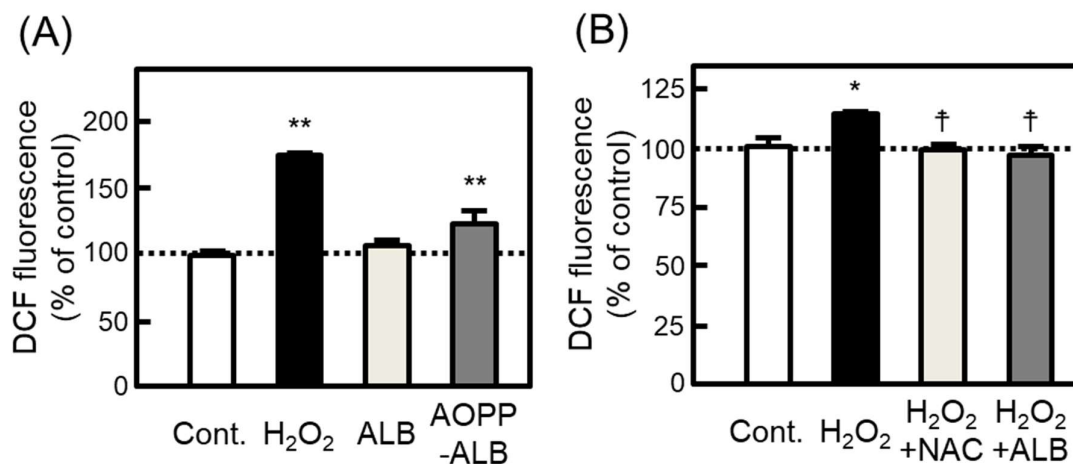


Figure 5. Effect of albumin and AOPP-albumin on ROS generation in A549 cells. (A) The cells were incubated with H₂O₂ (1 mM), albumin (5 mg/mL), or AOPP-albumin (5 mg/mL) for 24 h. (B) The cells were incubated with H₂O₂ (0.5 mM) for 24 h, then incubated with NAC (10 mM) or albumin (40 mg/mL) for 2.5 h. After the above-mentioned treatments, intracellular ROS levels were detected by measuring the fluorescence of 7'-dichlorofluorescein (DCF) using flow cytometry. Each value represents the mean ± SEM (n = 3). Significant differences from the values found in the control cells are indicated as * *p* < 0.05. Significant differences from the values found in the H₂O₂-treated cells are indicated as † *p* < 0.05.

Several types of scavenger receptors, including human scavenger receptor class A (SR-A), which binds LDL, and CD36 are shown to bind to modified albumin (24, 25). Iwao et al. clearly demonstrated the specific cell association of ^{125}I -AOPP-HSA in Chinese hamster ovary (CHO) cells with overexpression of CD36, but not in mock-CHO cells (26). In addition, Marsche et al. clarified that AOPP-albumin interferes with clearance of plasma HDL cholesterol mediated by SR-B1 in mice (27). However, in this study, knockdown of CD36 and SR-B1 in A549 cells had no effect on the FITC-AOPP-albumin uptake. Therefore, other types of scavenger receptors are likely to be involved in AOPP-albumin uptake into A549 cells. As oxidized albumin is known to contain a structural motif similar to advanced glycation end products (AGEs), the receptors associated with AGEs may be involved in AOPP-albumin transport into A549 cells (28, 29). Further studies are needed to identify the receptor responsible for transport of AOPP-albumin into A549 cells.

In this study, we found that FITC-AOPP-albumin uptake was inhibited by a clathrin-mediated endocytosis inhibitor, while caveolae-mediated endocytosis and micropinocytosis inhibitors did not affect the uptake of FITC-AOPP-albumin into A549 cells. Therefore, clathrin-mediated endocytosis is mainly associated with AOPP-albumin transport into A549 cells. We previously demonstrated that albumin was taken up by clathrin-mediated, but not by caveolae-mediated, endocytosis into A549 cells (10). In addition, albumin uptake into A549 cells was almost completely inhibited by AOPP-albumin (data not shown), indicating that AOPP-albumin may be taken up by a similar transport system to that for albumin. Although the association of SR-B1 with AOPP-albumin was not observed in this study, the shared binding site on SR-B1 for albumin and AOPP-albumin has been observed (27). Therefore, there may be a common endocytosis receptor with a site that can bind to both albumin and AOPP-albumin.

In immortalized rat intestinal epithelial cells, AOPPs induced ROS generation, followed by epithelial cell death, with an increase in expression level of apoptosis-related proteins and phosphorylation of c-jun N-terminal kinase (30). In this study, we showed that AOPP-albumin enhanced ROS generation in A549 cells, which would aggravate lung diseases, such as ARDS.

To evaluate the inhibitory effect of native albumin on H_2O_2 -induced ROS generation, we performed post-treatment of A549 cells with native albumin after H_2O_2 treatment and found an antioxidant effect of native albumin in A549 cells. The method used was based on a previous study showing that co-incubation of albumin with H_2O_2 induced a great loss of free thiols in albumin after 75 min (31). We also observed that co-treatment with native albumin had no effect on the H_2O_2 -induced increase in ROS generation (data not shown).

One of the important roles of serum albumin is its redox buffering action. In albumin, the Cys34 residue with free thiol has significant antioxidant activity. Thus, the ratio of the oxidized/unoxidized form of albumin, a function of the redox state of Cys34, could serve as one of effective markers for monitoring disease progression and therapeutic efficacy (32). The antioxidant effect of albumin, seen in this study, may induce Cys34 oxidation in albumin and the detection of the modified albumin in bronchoalveolar lavage fluid (BALF) could aid in diagnosis of several lung diseases.

In the acute lung injury mouse model developed by exposing to aerosolized liposaccharide, increased levels of ROS and albumin were detected in the BALF (33). In addition, albumin is known to be oxidized by ROS, producing AOPP-albumin (9). These facts suggest that conversion of albumin to AOPP-albumin by ROS would occur under *in vivo* conditions. Therefore, AOPP-albumin clearance from alveolar space should be important not only to enhance excess fluid clearance from the lung, but also to prevent the progression of lung disease. Further studies are necessary to clarify the detailed mechanism of AOPP-albumin transport and its physiological and pathophysiological role in lung alveoli.

CONCLUSION

The uptake of AOPP-albumin in the alveolar epithelial cell line, A549, was examined. FITC-labeled AOPP-albumin uptake in A549 cells was time- and temperature-dependent, and saturable. In addition, we found that FITC-AOPP-albumin uptake was mediated by clathrin-mediated endocytosis into A549 cells, but not by caveolae-mediated endocytosis or macropinocytosis. Furthermore, the present results showed that AOPP-albumin increased ROS

generation, while albumin had an antioxidant effect, indicating the importance of AOPP-albumin clearance from alveolar space. Although identification of AOPP-albumin receptor(s) was not achieved in this study, it is important to further clarify the molecular mechanisms underlying AOPP-albumin transport into alveolar epithelial cells.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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