Novel Delivery of Antioxidant Enzyme Catalase to Alveolar Macrophages by Fc Receptor-Mediated Endocytosis

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Excessive production of reactive oxygen species by alveolar macrophages (AMs) in response to inhaled toxic substances is a major cause of oxidative lung injury. Therapeutic approaches designed to protect the lungs from oxidative injury by administering native antioxidant enzymes such as catalase and superoxide dismutase have been suggested. However, problems associated with poor penetration of these enzymes to the intracellular target sites have limited their effective use. The present study reports a drug targeting method based on receptor-mediated endocytosis of the antioxidant enzyme catalase to the AMs. This method employs molecular conjugate consisting of a cognate moiety, in this case IgG which recognizes the macrophage Fc receptor, covalently linked to the enzyme catalase via the reversible disulfide linkage. The uptake efficiency of the enzyme conjugate and its protection against oxidative injury were evaluated microfluorometrically using the intracellular oxidative probe dichlorodihydrofluorescein BSA: anti BSA antibody complex (DCHF-IC), and the cell viability indicator propidium iodide. The DCHF-IC-stimulated macrophages exhibited a dose- and time-dependent increase in intracellular fluorescence with a half maximal response dose of approximately 120 μg/ml. Free catalase (50–500 U/ml) failed to inhibit the DCHF-IC-induced oxidative burst and had only a marginal protective effect on AM injury. In contrast, the catalase-IgG conjugate (50–500 U/ml) strongly inhibited both the DCHF-IC-induced oxidation and injury in a dose-dependent manner. Effective inhibition was shown to require both the antioxidant catalase moiety and the cognate moiety for the cell surface receptor. Specific internalization of the conjugate through the Fc receptor was also investigated by competitive inhibition using free IgG. Under this condition, the conjugate showed a much reduced protective effect on intracellular oxidation, indicating conjugate internalization through the Fc endocytosis pathway. Thus, the enzyme-IgG conjugate system may be used as an effective and selective means to deliver antioxidant enzymes to the intracellular oxidative targets of the AMs.

KEY WORDS: macrophages; immune complex; catalase; oxidation; endocytosis.

INTRODUCTION

Immune reactions in the lungs often result in pulmonary inflammation, injury, and fibrosis (1). These reactions involve release of, or reaction to, reactive oxygen species (ROS), either exogenous as in pollutants, or endogeneous as in products of phagocytic macrophages. Macrophages produce ROS in response to various stimuli via the activation of NADPH oxidase complex. The complex is internally regulated and uses NADPH to reduce molecular oxygen to superoxide and other oxidative products (2). The lungs are normally protected against the destructive nature of these ROS by a variety of mechanisms, most notably the intracellular antioxidant enzyme catalase and superoxide dismutase which scavenge hydrogen peroxide and superoxide anion respectively (3,4). In disease states like pulmonary injury and fibrosis, these endogenous enzymes are overwhelmed, and unchecked ROS destroy healthy tissue, leaving behind inelastic scar tissue which is practically impermeable and therefore can not participate in the blood-gas exchange function of the lung.

Delivery of antioxidant enzymes directly into pulmonary tissue to augment the endogenous enzymes would, therefore, serve to neutralize excess ROS and help defend against further lung damage. However, poor accessibility of these enzymes to the intracellular target sites of pulmonary cells has greatly limited their effective use (5). Strategies for enhanced therapeutic delivery of antioxidant enzymes have, thus far, focused on the use of the liposome entrapment systems. These systems have permitted intracellular access of the enzyme macromolecules and help protect the lung from oxidative injury (6,7). However, these systems generally lack cellular specificity and, in some cases, can cause cellular toxicity when used at high doses (7). As an alternative strategy to circumvent these problems, the present study investigates the feasibility of delivering antioxidant enzyme directly to the AM by means of receptor-mediated endocytosis. The AM is targeted because it is the major source of ROS production in the lung (1). To accomplish enzyme delivery, the study utilizes a molecular conjugate consisting of catalase, covalently-linked to IgG, a cognate moiety for the Fc macrophage receptor (8). When recognized by the receptor, the conjugate is internalized by the efficient receptor-mediated pathway, co-transporting the enzyme. Unlike other existing methods of delivery, the method described here directly targets AM and delivers the enzyme to the major site of ROS production, i.e., in the phagosomes (2,9). Thus, the method has the potential to be more effective and perhaps less toxic to the surrounding cells and tissues. To evaluate the effectiveness of this method, a phagocytic oxidative probe, dichlorodihydrofluorescein-bovine serum albumin (BSA): anti-BSA antibody complex was used to stimulate the AM, and the inhibitory effects of the IgG-catalase conjugate on intracellular oxidation and cell injury were then studied.

MATERIALS AND METHODS

Alveolar Macrophage Preparation

The AMs were harvested from male Sprague-Dawley rats (200–250 gm) by bronchoalveolar lavage. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (0.2 gm/kg body weight). The trachea was cannu-

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lated and the lungs lavaged 10 times with 8 ml aliquots of Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4). Lavage cell suspensions were centrifuged at 500*g* for 5 min at 4°C. The cell pellets then were washed twice by alternate resuspension and centrifugation in HEPES-buffered medium (136 mM NaCl, 2.2 mM Na_2HPO_4, 5.3 mM KCl, 10 mM HEPES, 5.6 mM glucose and 1 mM $CaCl_2$, pH 7.4). Cell number, purity, and mean cell volume of AM preparations were determined using a Coulter model Z_B electronic cell counter with a cell sizing attachment (Coulter Instrument, Hialeah, FL). The average values for these parameters were: yield = $5.8 \pm 0.2 \times 10^6$ AMs/rat; purity = $91.5 \pm 0.5\%$; and mean cell volume = $1{,}410 \pm 10$ μm^3. Cell viability was measured via trypan blue exclusion and was found to be >95%. One hundred microliter aliquots containing 1×10^6 cells/ml were added onto tissue culture cover slips (Corning, NY), placed in flat-bottomed 12 well plates, and the cells were allowed to attract on the glass surface at room temperature for 1 hr prior to use.

Alveolar Epithelial Cell Preparation

Alveolar epithelial type II cells were prepared according to the method previously described (10). Briefly, rat lungs were lavaged to remove free AMs and were then excised and filled with phosphate-buffered saline (PBS) containing elastase (40 U/ml, type I, US Biochemical) and DNase (0.006%, Sigma). After a 20-min incubation period at 37°C, the lungs were finely minced and the digestion was arrested by incubation for 5 min in PBS containing 25% fetal bovine serum and 0.006% DNase (to help prevent cell clumping). The crude extract was sequentially filtered through 160 and 45 μm screens, centrifuged, and the resulting cell pellet was spun on the Percoll density gradient. The second cell band from the surface were collected, washed, and resuspended in HEPES-buffered medium. The cell suspension yielded 15–20 $\times 10^6$ cells/rat with viability generally greater than 95%.

Synthesis of Immunoglobulin-Catalase Conjugate

Rat IgG and catalase (EC 1.11.1.6) were obtained from Sigma Chemical Company (St. Louis, MO). The IgG was isolated from pooled normal rat serum by fractionation and ion-exchange chromatography. The two were coupled by ligation through disulfide bond after chemical modification with the bifunctional cross-linking reagent N-succinimidyl 3-(2-pyridyldithio) proprionate (SPDP), according to the modified method of Carlsson *et al.* (11). The IgG (0.05 μmol) was dissolved in 0.5 ml of 0.1 M PBS, pH 7.4. An ethanol solution (5 μl) containing 10 mM SPDP was added to the stirred protein solution and the reaction proceeded for 30 min at room temperature. Excess of the reagent was removed by centrifugal filtration through a dialysis membrane filter (Durapore™ CL5K, Millipore Corp.) at 5,000 g for 30 min. The modified protein was then collected and reconstituted in 0.5 ml sodium acetate buffer, pH 4.5. Modification of the catalase was similarly performed except that the protein was reconstituted in PBS. The IgG-2-pyridyl disulfide derivative obtained was then converted into a thiol derivative by specific reduction of the 2-pyridyl disulfide groups with dithiothreitol (10 μmol) for 30 min, after which it was centrifuged and reconstituted in 0.5 ml PBS. The modified IgG was then mixed with the catalase-2-pyridyl disulfide derivative and, after a 18 hr incubation period at 4°C, the reaction mixture was centrifugally filtered through the Durapore™ CL300K membrane to remove unreacted proteins. The IgG-catalase conjugate was then collected and stored at 4°C in HEPES-buffered medium for further studies. Characterization of the conjugate was performed using gel permeation chromatography (Pharmacia Superpose 12 column) with the aid of protein molecular weight markers (12). This study showed that the conjugate obtained was a monoconjugate. Analysis of catalase activity was performed by measuring the decomposition of H_2O_2 at A_{240} (13). One unit of catalase is the amount of enzyme that decomposes 1 μmol H_2O_2 per min. The conjugate prepared under these conditions contains approximately 90–100 U/μg total protein as determined by micro Lowry Assay.

Measurement of Intracellular Oxidation

Stimulation and measurement of intracellular oxidation was achieved by treating the cells with the IgG immune complex (DCHF-IC) which consists of the dichlorodihydrofluorescein BSA: anti-BSA IgG antibody complex (Fc-Oxyburst™, Molecular Probes Inc., Eugene, OR). Once the immune complex is endocytosed through the Fc receptor, the nonfluorescent oxidative probe dichlorohydrofluorescein is oxidized to form a green fluorescent product, dichlorofluorescein. This fluorescence is then measured by the Spex ARCM detection system (Spex Ind., Edison, NJ) which is attached to the Nikon Diaphot microscope. Measurements were conducted at 37°C on the thermal microscope stage at the excitation and emission of 490 and 520 nm respectively. The Oxyburst™ immune complex was used in this study because it provides a rapid, sensitive, and simultaneous stimulation and detection of the oxidative burst in living cells (14).

Determination of Cellular Damage

The AMs were incubated at 37°C in HEPES-buffered medium consisting 1 μg/ml propidium iodide. At appropriate time intervals following immune complex treatments, cellular fluorescence signals were detected at the excitation and emission wavelengths of 490 nm and 600 nm respectively. Propidium iodide, due to its hydrophilicity, is normally excluded from intact cells, but, if the cell membrane is damaged, the probe can enter the cell and bind specifically to the cell nucleus. Upon binding, its fluorescence intensity is strongly enhanced; therefore intense nuclear fluorescence indicates membrane damage and cell death (14). In these experiments, Triton X-100 (0.1%) was used to permeabilize the cells in order to establish maximal fluorescence signal. Cell damage was estimated from the maximum and minimum (baseline) fluorescence signals according to the equation:

$$\% \text{ Damaged Cells} = \frac{\text{Measured signal} - \text{Minimum signal}}{\text{Maximum signal} - \text{Minimum signal}} \times 100\%$$

RESULTS AND DISCUSSION

Effect of Immune Complex on Intracellular Oxidation

Upon exposure to the immune complex (60–480 μg/ml), the AMs demonstrated a dose- and time-dependent increase in intracellular fluorescence (Fig. 1). The cell response was biphasic with an initial fluorescence rise that occurred before the first accessible time point, i.e., within seconds after stimulus exposure, followed by a sustained fluorescence increase which remained linear throughout the examination period. Cellular autofluorescence was minimal and did not interfere with the assay. These observations are consistent with previous findings in human neutrophils by Ryan *et al.* (15) which demonstrated the similar biphasic characteristic of the cellular response to the DCFH immune complex. In that study, the initial fluorescence response was attributed to the binding of the complex to the cells whereas the subsequent fluorescence rise was associated with the actual oxidative event in the cells. To further confirm these findings in AMs, we conducted the experiments in which the cells were exposed to the immune complex at 4°C, the condition known to inhibit endocytic uptake but not binding. Under this condition, the cells exhibited only the initial fluorescence response but not the secondary response (results not shown), thereby supporting the earlier findings.

To further characterize the binding and oxidation dependence of the fluorescence changes, rat alveolar epithelial cells which do not possess Fc receptors and are incapable of mounting an oxidative burst, was also tested. The results of this study showed that in contrast to the AMs, the alveolar epithelial cells exhibited much reduced cellular fluorescence response in both phases (Fig. 2), indicating that the immune complex indeed mediated its effect through the binding and activation of the Fc receptor. The results also demonstrated preferential uptake of the immune complex, and presumably the IgG-catalase conjugate in subsequent studies, via the endocytic process by the AMs over the epithelial cells.

Enzyme Delivery to Macrophages Mediated by IgG-Catalase Conjugate

To determine the feasibility of delivering the antioxidant enzyme catalase to the AMs via the Fc receptor-mediated endocytosis, the cells were preexposed to the IgG-catalase conjugate (50–500 U/ml) and their fluorescence intensities in response to the immune complex (120 μg/ml) were monitored. As a comparison, free catalase (at equivalent activities) was also used and its effect on intracellular fluorescence was analyzed in parallel. The results, shown in Fig. 3, indicated that free catalase had no significant effect on cellular fluorescence response (at all concentrations used) whereas the IgG-catalase conjugate significantly inhibited the fluorescence signals in a dose-dependent manner, i.e., 88%, 76%, and 54% reduction at the conjugate concentrations of 50, 250, and 500 U/ml respectively. Thus, our results clearly demonstrated the effectiveness of this conjugate system as a means to deliver the enzyme for the protection against in-

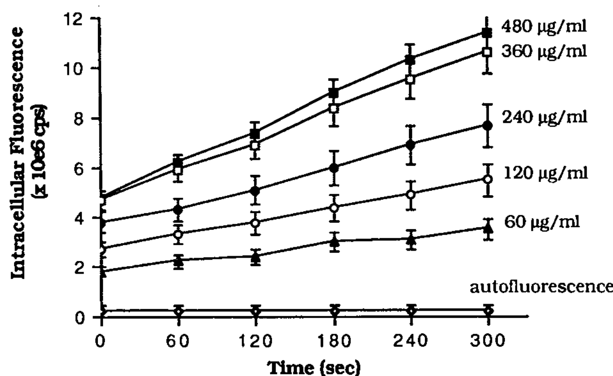

Figure 1 *Stimulus Dose Response of DCFH-IC-Activated Rat Alveolar Macrophages.* Cells (1×10^6/ml) were exposed to varying concentrations of DCFH-IC (60–480 μg/ml) in HEPES-buffered medium and their intracellular fluorescence signals were monitored as a function of time. The values represent mean ± SE for four measurements obtained from different cell preparations.

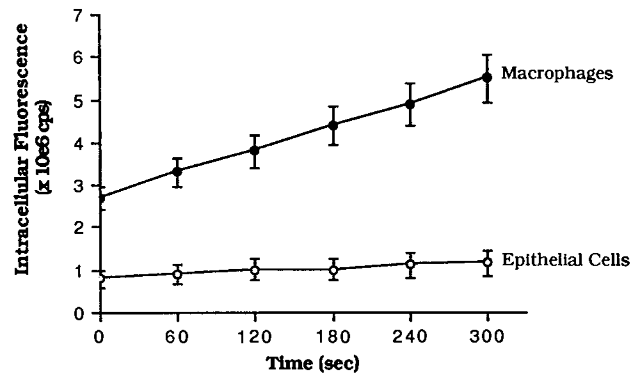

Figure 2 *Comparison of Cellular Response to Immune Complex in Alveolar Macrophages and Epithelial Cells.* Isolated cell preparations of either macrophages or epithelial cells (1×10^6/ml) were incubated with DCFH-IC (120 μg/ml) in HEPES-buffered medium. The values represent mean ± SE for four measurements obtained from different cell preparations.

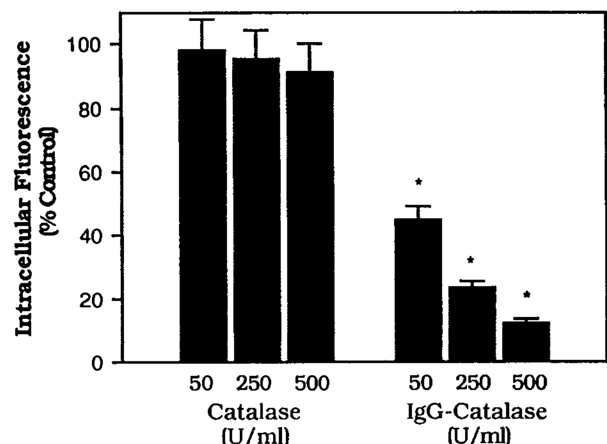

Figure 3 *Dose Effects of Catalase and IgG-Catalase Conjugate on Macrophage Activation-Dependent DCFH-IC Oxidation.* Cells were preincubated with varying concentrations of free catalase (50–500 U/ml) or IgG-catalase conjugate (50–500 U/ml) for 15 min in HEPES-buffered medium and their responses to DCFH-IC (120 μg/ml) were monitored. The data represent percent control values (mean ± SE, n = 4). The control is the fluorescence response obtained 5 min after treating the cells with the DCFH-IC in the absence of catalase or catalase conjugate. *Significant decrease from the control ($p < 0.05$).

tracellular oxidation. The observation of the protective effect of the catalase conjugate also indicated the role of hydrogen peroxide as a major oxidative species involved in the immune complex-mediated oxidative burst in AMs. The failure of free catalase to inhibit intracellular oxidation can be attributed to its inaccessibility to the intracellular target sites, e.g., phagosomes, which is consistent with previous findings both *in vivo* and *in vitro* (5–7,15). Further, because catalase was shown to be capable of inhibiting intracellular oxidation of free dichlorodihydrofluorescein (DCFH) which localized in the cytoplasm but not the phagosomes (15), our study also suggested that the immune complex-stimulated oxidative burst occurred predominantly in the phagocytic vacuoles of the cells. Thus, targeted delivery of the antioxidant enzyme to these organelles by the immune conjugate provides a logical approach towards the protection against immune complex-stimulated oxidation.

Mechanism of Enzyme Uptake Mediated by IgG-Catalase Conjugate

To further confirm that the enzyme conjugate was taken up phagocytically via the Fc receptor, the contribution of the IgG moiety of the conjugate in affecting enzymatic uptake was evaluated. The conjugate alone (500 U/ml or ≈5 μg/ml), or in combination with excess IgG (0.01–0.1 mg/ml, to provide competitive inhibition for the Fc receptor), or IgG alone (0.1 mg/ml), was evaluated for their ability to inhibit the cellular response to the immune complex (Fig. 4). In this study, the conjugate alone inhibited the oxidative response much greater than did the conjugate in the presence free IgG. Increasing the amount of the IgG in the incubating medium lessened the inhibitory effect of the conjugate on the cellular oxidative response, i.e., 44% and 13% reduction in the presence of 0.01 and 0.1 mg/ml IgG, as compared to 55% reduction in the absence of IgG. IgG alone had no significant effect on cellular fluorescence response, indicating that the inhibitory effect of the immune conjugate requires the presence of catalase. Thus, our results are consistent with the concept that enzyme uptake mediated by the conjugate requires functional domain capable of cell-surface receptor recognition.

Protection Against Oxidative Injury by IgG-Catalase Conjugate

Excessive exposure to immune complexes is known to induce ROS-mediated lung injury (16–18). Consistent with these findings, our results based on fluorescence propidium iodide studies also indicated that the DCFH-IC (120 μg/ml) can induce AM damage in a time-dependent manner, i.e., 9%, 21%, and 31% of the total cells were damaged after 0.5, 1.0, and 1.5 hr respectively (Fig. 5). Control studies using untreated cells showed minimal AM damage, i.e., <3% after 1.5 hr. Pretreatment of the cells with free catalase (50 U/ml) had a marginal protective effect on cell injury at least during the first hour, consistent with earlier oxidative studies. However, at 1.5 hr, catalase partially but significantly inhibited AM damage caused by the immune complex (20%). This latter observation suggests that, in addition to activating intracellular ROS production, the immune complex also stimulated, to a lesser extent, extracellular ROS secretion which, upon accumulation, can potentiate cell damage. Additionally, stimulated ROS in the phagocytic vacuoles may also leak into the cytoplasm after a delayed period, which can then be scavenged by the catalase (15). In contrary to free catalase, the IgG-catalase conjugate (50 U/ml) was much more effective in protecting the cells from oxidative damage, i.e., only 8% of total cells were damaged after 1.5 hr (compared to 31% and 20% in untreated and catalase-treated cells, respectively). This result again agreed well with our earlier oxidative studies which indicated enhanced oxidative inhibition by the conjugate. Similarly, IgG alone had no significant effect on oxidative cell damage (data not shown). Taken together, our results indicate that the enzyme-IgG conjugate may be used to enhance therapeutic uptake of the enzyme for the protection against oxidative AM injury. It

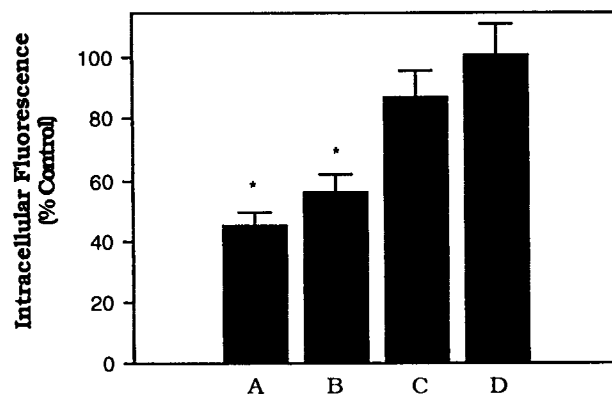

Figure 4 *Mechanism of Enzyme Delivery to Alveolar Macrophages Mediated by IgG-Catalase Conjugate.* Cells were treated with DCFH-IC (120 μg/ml) in the presence of (A) 5 μg/ml IgG-catalase conjugate, or (B) same as A + 0.01 mg/ml IgG, or (C) same as A + 0.1 mg/ml IgG, or (D) 0.1 mg/ml IgG alone. The data represent percent control values (mean ± SE, n = 4). The control is the fluorescence response obtained 5 min after immune complex stimulation. *Significant decrease from the control ($p < 0.05$).

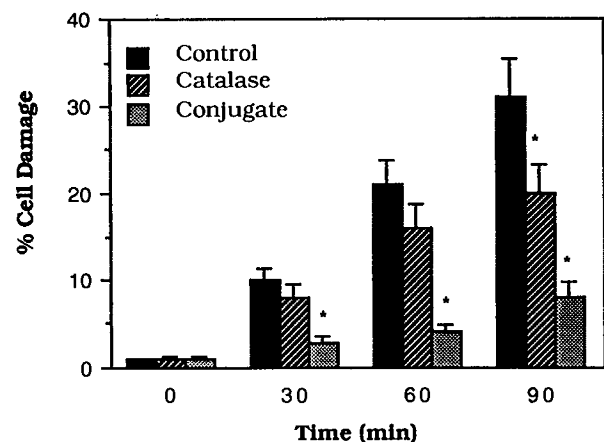

Figure 5 *Effect of Immune Complex-Induced Intracellular Oxidation on Macrophage Injury.* Cells were incubated with 120 μg/ml DCFH-IC in the absence (control) or presence of 50 U/ml catalase, or 50 U/ml IgG-catalase conjugate. Cellular damage was monitored using fluorescence propidium iodide assay as described in the text. The data represent percent control values of the unstimulated cells (mean ± SE, n = 4). *Significant decrease from the immune complex controls ($p < 0.05$).

should be noted that while our studies indicated that hydrogen peroxide was the major ROS involved in AM injury, other metabolic products of hydrogen peroxide, i.e., the highly reactive hydroxyl radical (OH·) which could be generated via the iron-mediated Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow OH \cdot + Fe^{3+} + OH-$) may be responsible for the actual cell damage. In this regard, it is of interest to note that iron chelators such as deferoxamine were also effective in preventing oxidative damage caused by the IgG-immune complexes (19).

In summary, our studies demonstrated that the immune complex-induced ROS production in AMs occurred predominantly in the phagocytic vacuoles. This compartmental oxidative event disallows direct contact between the exogenously administered catalase and the ROS which, in turn, limits the effectiveness of catalase as an antioxidant and as a protectant against the immune complex-induced AM injury. Enhanced therapeutic efficiency of this enzyme can be accomplished by chemical conjugation of the enzyme with the phagocytic vector IgG. The selectivity of this method to Fc bearing cells such as AMs and neutrophils, both of which play an important role in oxidative lung injury, as well as its ability to target the localized ROS in the phagocytic vacuoles make this method highly attractive as an effective means to deliver antioxidant enzymes to the lung.

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