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Original Research Communication

Ability of Antioxidant Liposomes to Prevent Acute and Progressive Pulmonary Injury

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ABSTRACT

We recently showed that acute oxidant-related lung injury (ALI) in rats after application of 2-chloroethyl ethyl sulfide (CEES) is attenuated by the airway instillation of antioxidants. We investigated whether intratracheal administration of antioxidant-containing liposomes immediately after instillation of CEES would attenuate short-term as well as long-term (fibrotic) effects of CEES-induced lung injury. In the acute injury model (4 h after injury), *N*-acetylcysteine (NAC)-containing liposomes were protective and reduced to baseline levels both the lung permeability index and the appearance of proinflammatory mediators in bronchoalveolar lavage fluids from CEES-exposed lungs. Similar results were obtained when rat alveolar macrophages were incubated *in vitro* with either CEES or lipopolysaccharide in the presence of NAC-liposomes. When lung fibrosis 3 weeks after CEES was quantitated by using hydroxyproline content, liposomes containing NAC or NAC + glutathione had no effects, but liposomes containing α/γ-tocopherol alone or with NAC significantly suppressed the increase in lung hydroxyproline. The data demonstrate that delivery of antioxidants *via* liposomes to CEES-injured lungs is, depending on liposomal content, protective against ALI, prevents the appearance of proinflammatory mediators in bronchoalveolar fluids, and suppresses progressive fibrosis. Accordingly, the liposomal strategy may be therapeutically useful in CEES-induced lung injury in humans. *Antioxid. Redox Signal.* 10, 973–981.

INTRODUCTION

Oxidant-mediated acute lung injury (ALI) is known to occur in a variety of conditions [including exposure to mustard gas and its derivatives such as 2-chloroethyl ethyl sulfide (CEES)] (8). CEES is a chemical agent that initiates cell injury associated with an imbalance between oxidants and antioxidants. On a subcellular level, it is known that sulfur mustard leads to an increase of gene expression involved in inflammation, apoptosis, and cell-cycle regulation (5). In addition to acute lung injury, lung exposure to sulfur mustard or its derivatives also leads to progressive lung injury, being represented by extensive interstitial fibrosis, which is well described in humans exposed to mustard gas compounds (18, 25) and in rats exposed to CEES (29). No specific therapy exists for the treatment of humans exposed to inhaled mustard gas compounds, although in the case of ALI in rats after exposure to CEES, we previously showed that protective strategies were associated with liposomes containing antioxidant enzymes (catalase, superoxide dismutase) or liposomes containing iron chelators or

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nonspecific reducing agents (30). When given at the time of lung exposure to CEES, these agents reduced the intensity of acute lung injury (ALI), as defined by albumin leak into lung, by as much as 70%. Delayed instillation of these liposomes for as long as 90 min was still protective. These findings are consistent with the hypothesis that CEES upsets the redox balance in lung, leading to a loss of reducing equivalents in the lung (23, 26).

In a recent study, we showed that liposomes containing antioxidants, such as GSH or α-tocopherol given intratracheally at the time of injury, were also protective against ALI, as measured 4 h after airway instillation of CEES (29). Collectively, our studies have suggested that ALI occurring after airway exposure to CEES is associated with an intense lung-damaging inflammatory response featuring involvement of the complement system and neutrophils [polymorphonuclear leukocytes (PMNs)], both of which intensified inflammatory injury. In other words, exposure of lung to CEES triggered a lung-damaging inflammatory response. Instillation into CEES-exposed lungs of antioxidant liposomes was remarkably protective, even when liposomal delivery was delayed for 90 min after CEES instillation, suggesting that time for effective treatment of lungs may exist after exposure to CEES. Finally, it was also shown that CEES exposure of rat lung led to pulmonary fibrosis, although the effectiveness of antioxidant liposomes in that setting is not known.

Lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, is known to trigger inflammatory reactions in lung similar to the effects of CEES (16, 51). Stimulation of macrophages by LPS results in activation of nuclear transcription factors [nuclear factor κB (NF-κB)] and release of proinflammatory cytokines (10, 20, 37, 38). Further, it has been shown that systemic administration of NAC may attenuate LPS-induced injury (15). In the context of CEES, LPS has been reported to enhance the cytotoxic effects of CEES (40). In the current study, we further explore the ability of antioxidant liposomes to protect against acute and progressive lung injury in rat lungs exposed to CEES. The data suggest that introduction of antioxidant interventions is protective in the lung.

MATERIALS AND METHODS

Chemicals

Except where noted, all chemicals and reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Animal model

Adult male (275–325 g) specific pathogen-free Long-Evans rats (Harlan Co., Indianapolis, IN) were used in these studies. Intraperitoneal ketamine (100 mg/kg body weight) (Fort Dodge Animal Health, Fort Dodge, IA) was used for anesthesia, and intraperitoneal xylazine (13 mg/kg body weight) (Bayer Corp., Shawnee Mission, KS) for sedation. The experimental procedure for CEES-induced lung injury in rats was described previously (30). In brief, after induction of anesthesia, the trachea was surgically exposed, and a slightly curved P50 catheter was inserted into the trachea past the bifurcation to facilitate a unilateral, left-lung injury. A small volume of CEES (2 μl/rat; ~6 mg/kg) was solubilized in ethanol (58 μl/rat) and then added to a syringe containing Dulbecco's phosphate-buffered saline (DPBS) (340 μl/rat). This solution was injected *via* the intratracheal catheter into the left-lung mainstem bronchus. Animals were killed at indicated time points by exsanguinations *via* the inferior vena cava. All animal experiments were in accordance with the standards in The Guide for the Care and Use of Laboratory Animals, approved by the University Committee on Use and Care of Animals (UCUCA), and were supervised by veterinarians from the Unit for Laboratory and Animal Care of the University of Michigan Medical School.

Permeability index

The lung permeability index was determined as described later. Studies not requiring the use of a radiolabeled marker (^{125}I-BSA) proceeded identically, substituting DPBS (intravenously) for the radiomarker. For experiments where indicated, 0.5 mg bovine serum albumin (BSA) was mixed with 0.5 μCi ^{125}I-BSA, and the material (in 0.5 ml) was injected intravenously into rats. Animals were killed 4 h later, and the pulmonary arterial circulation was flushed with 10 ml of cold DPBS. The lungs were then surgically dissected, and the amount of radioactivity (^{125}I-labeled BSA) in lung parenchyma determined by gamma counting. For calculations of the permeability index, the amount of radioactivity remaining in perfused lungs was divided by the amount of radioactivity present in 1.0 ml of blood obtained from the inferior vena cava at the time of death, as described elsewhere (30). ^{125}I-BSA present in lung is a quantitative measure of the degree of vascular endothelial and alveolar epithelial damage, in which much of the ^{125}I-BSA can be lavaged from the distal airway compartment, indicating loss of the vascular and epithelial barrier function (24).

Liposome preparation

Dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids, Alabaster, AL) was dissolved 20 mg/ml in a 2:1 vol/vol chloroform/methanol solution. The DPPC solution was then dried under a thin stream of nitrogen in a round-bottom flask to form a thin lipid film on the walls of the tube. Once the film had been dried, the tube was then placed on a vacuum for at least 1 h to dry further and to remove any excess organic compounds from the lipid film.

The following compounds, being encapsulated within the liposomes, were prepared in Dulbecco's phosphate-buffered saline (DPBS), pH adjusted to 7.4. *N*-Acetylcysteine (NAC, 30 mg/ml), glutathione (GSH, 30 mg/ml), vitamin E (α + γ-tocopherol, each at 1.3 mg/ml) were then added to the lipid film. The tube was then vortexed to free the lipid film from the walls of the tube and then placed in a heated water bath (41°C). When sizing the liposomes, it was necessary to keep them at a temperature above their transition phase (41° C). Vortexing the liposomes once they were above the transition-phase temperature resulted in large multilamellar vesicles. To reduce the size of the liposomes and to produce uniform small unilamellar vesicles, the lipid suspension was then passed 10 times through polycarbonate membrane filters in a Liposofast Basic miniextruder (Avestin, Inc., Ottawa, Ontario, Canada). The resulting liposomes were uniform in size, measuring 100 nm in diame-

ter. It is well known that the pharmacokinetics of liposome uptake is dependent on particle-size distribution (11, 12). Uniformity and size of the liposomes was confirmed by light microscopy. Liposomes were injected intratracheally (volume of 100 μl per rat) through the same catheter setup immediately after the CEES instillation. This translates into 3 mg NAC, 3 mg GSH, and 0.13 mg α- or γ-tocopherol, respectively (the same doses apply where combinations of antioxidant agents were given). The same conditions were used when soluble NAC was used. *N*-Acetylcysteine (NAC, 30 mg/ml) was prepared in Dulbecco's phosphate-buffered saline (DPBS); pH adjusted to 7.4 and 100 μl of the suspension per rat was injected intratracheally.

Cytokine measurements in bronchoalveolar lavage fluid

To determine the concentration of various inflammatory cytokines in the lungs, bronchoalveolar lavage fluids (BALFs) were obtained selectively from the left, (injured) lung lobe at indicated time points after administration of CEES. After centrifugation, IL-1β, IL-6, TNF-α, and CINC-1 were measured in the supernatant fluids by using commercially available ELISA kits and by following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

In vitro *stimulation of alveolar macrophages*

Alveolar macrophages were obtained from normal rat lungs by bronchoalveolar lavage. After pooling, gentle centrifugation and counting of the cells (with a yield of $\sim 10 \times 10^6$ cells per lung), macrophages resuspended in RPMI medium containing 0.5% BSA were incubated for 1 h to allow for settling. To create an *in vitro* situation similar to the *in vivo* CEES model, 2.5×10^6 cells/ml media were incubated with 500 μM CEES with or without antioxidant liposomes (50 μl) for 4 h. In additional experiments, plated macrophages were incubated with 100 ng/ml lipopolysaccharide (LPS) from *Escherichia coli* (serotype O111:B4) and 50 μl liposomes. After centrifugation, IL-1β, IL-6, TNF-α, and CINC-1 were measured in the supernatants by using commercially available ELISA kits and by following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Hydroxyproline assays

Hydroxyproline is a modified amino acid found at a uniquely high percentage in collagen. Therefore, we determined hydroxyproline content of the lungs as a quantitative measure of collagen deposition, as described previously. Although the accuracy of this assay may potentially be affected by proteins containing collagen-like motifs (*e.g.*, surfactant), its usefulness for determining tissue collagen content has been established and well accepted (31). Rats were killed 3 weeks after exposure to CEES, and the pulmonary circulation was flushed with 10 ml cold DPBS. The left (injured) lung was surgically removed. The isolated lobes were homogenized in 1 ml of PBS, and hydrolyzed by the addition of 1 ml of 12N HCl. Samples were then baked at 110°C for 12 h. Aliquots were then assayed by adding chloramine-T solution for 20 min followed by development with Ehrlich's reagent at 65°C for 15 min, as previously described (47). Absorbance was measured at 550 nm, and the amount of hydroxyproline was determined against a standard curve generated by using known concentrations of pure hydroxyproline.

Morphologic assessment of lung injury

Morphologically to assess lung injury, lungs were fixed by gentle intratracheal instillation of 10 ml buffered (pH 7.2) formalin (10%) at the indicated time points after airway instillation of CEES. Tissues were embedded in paraffin. Lung sections were then obtained for histologic examination by using hematoxylin and eosin stain. In addition, lung sections were stained with Masson trichrome to assess deposition of fibrin and collagen (28).

Statistical analysis

Results are presented as mean ± SEM in the text and figures. Groups ($n = 5$ rats) were subjected to one-way analysis of variance (ANOVA), and when significance was found, Tukey's *post hoc* test was applied. A value of $p < 0.05$ was considered significant.

RESULTS

Protective effects of NAC-containing liposomes in ALI

Because NAC-containing liposomes are known to protect against acute (4-h) CEES-induced injury in rat lungs when

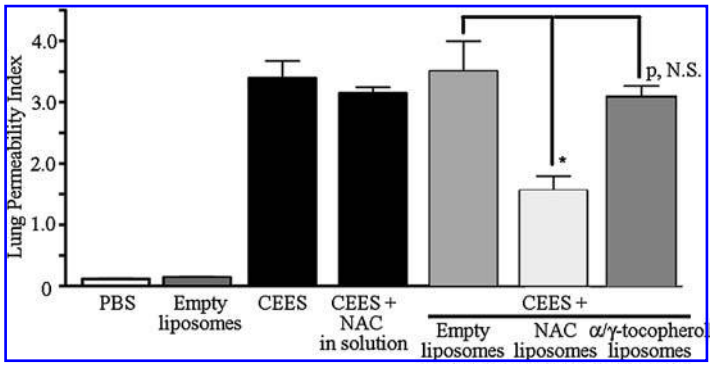

FIG. 1. Permeability indices in rat lungs 4 h after airway delivery of PBS (control) or empty liposomes, or CEES (6 mg/kg) in the absence of liposomes or in the presence of empty liposomes, NAC-containing (30 mg/ml), or α/γ-tocopherol-containing (1.3 mg/ml) liposomes. An additional group of animals received soluble NAC dissolved in PBS (fourth bar, CEES + NAC in solution) by using the same conditions used for preparation of NAC-liposomes. The permeability index was the ratio of ^{125}I-albumin in left lung parenchyma to the radioactivity present in 1.0 ml blood (vena cava) obtained at the time of death. For each bar, $n = 6$ animals. *$p < 0.05$.

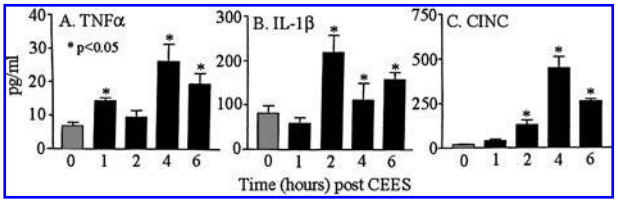

FIG. 2. Appearance of TNF-α, IL-1β, and CINC-1 (as measured by ELISA) in BAL fluids from the left (injured) lung as a function of time after intratracheal instillation of CEES into rat lungs. For each bar, $n = 6$ animals. *$p < 0.05$ when compared with 0 time values.

given intratracheally immediately after instillation of CEES (29), experiments were designed to assess the protective effects of empty liposomes, NAC in solution (PBS), NAC-liposomes, and α/γ-tocopherol liposomes. As shown in Fig. 1, control lungs (receiving PBS or empty liposomes) showed very little evidence of albumin leak (permeability index), with values <0.15. CEES caused an intense leak of serum albumin, with the permeability index ~3.4, whereas CEES instillation followed by NAC in PBS (pH 7.4) showed a very modest reduction in the permeability index (<10%). Empty liposomes or liposomes loaded with α/γ-tocopherol, given into the upper airways immediately after CEES, did not reduce the permeability index (same as with CEES alone), whereas in the case of NAC-liposomes, a 59% reduction in the permeability index was found ($p < 0.05$).

Time course for mediators in BAL fluids after CEES

To establish the time course for appearance of inflammatory mediators in BAL fluids from rats given CEES into the left lung at time 0, BAL samples were obtained at 0, 1, 1.5, 2, 4, and 6 h after CEES administration. Mediators were measured with ELISA (Fig. 2). Time courses for TNF-α, IL-1β, and CINC-1 indicated that, as a general rule, these proinflammatory mediators peaked between 2 and 4 h after instillation of CEES into rat lungs. For subsequent experiments, the 4-h time point was selected.

Reductions in BAL Mediators in CEES lungs after antioxidant liposomes

We previously showed that liposomes containing NAC prevent the ALI that follows airway administration of CEES (30). CEES was used to induce ALI in the absence of liposomes or in the presence of empty liposomes or NAC-containing liposomes, the last of which are known to be protective in the ALI CEES model (29). In the current studies, we sought to determine to what extent protective liposomes would affect BAL levels of cytokines and chemokines. BAL fluids were obtained 4 hours after airway administration of CEES in the absence or presence of empty liposomes or NAC-liposomes, as indicated in Fig. 3. CEES caused ~20-fold, fourfold, 2.5-fold, and 1.6-fold increases in CINC-1, IL-1β, IL-6, and TNF-α, respectively, in BAL fluids. The presence of empty liposomes with CEES caused little or no change when compared with the values found with CEES alone. Conversely, NAC-liposomes caused the levels of BAL mediators to decrease to virtually baseline levels in all cases.

Effects of antioxidant liposomes on in vitro *production of cytokines and chemokines by lung macrophages*

Two different *in vitro* experiments were used in which alveolar macrophages (2.5×10^{-6}/ml) from normal rat lungs were

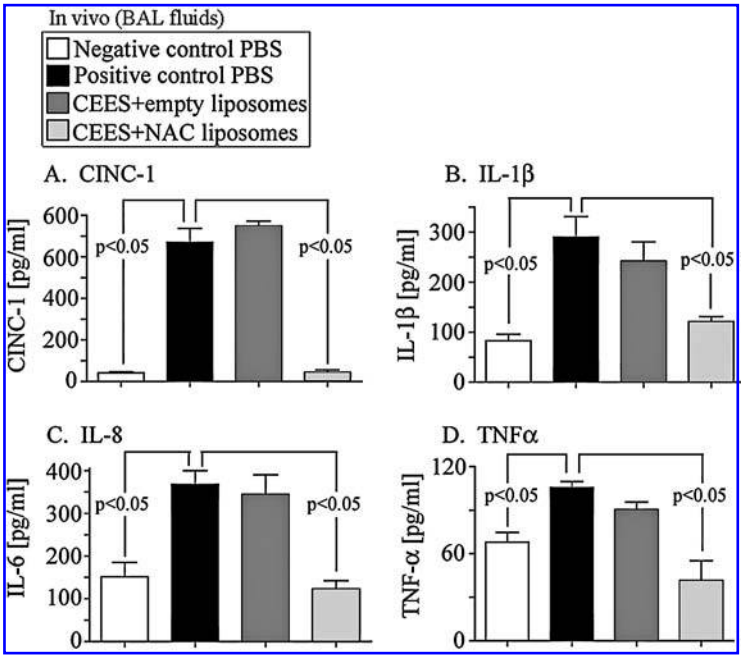

FIG. 3. Levels of rat CINC-1 (A), IL-1β (B), IL-6 (C), and TNF-α (D) in BAL fluids from left lung 4 h after instillation of PBS (control) or CEES. As indicated, CEES was instilled, followed by airway instillation of empty liposomes (lips) or NAC-liposomes. For each bar, $n = 8$ animals.

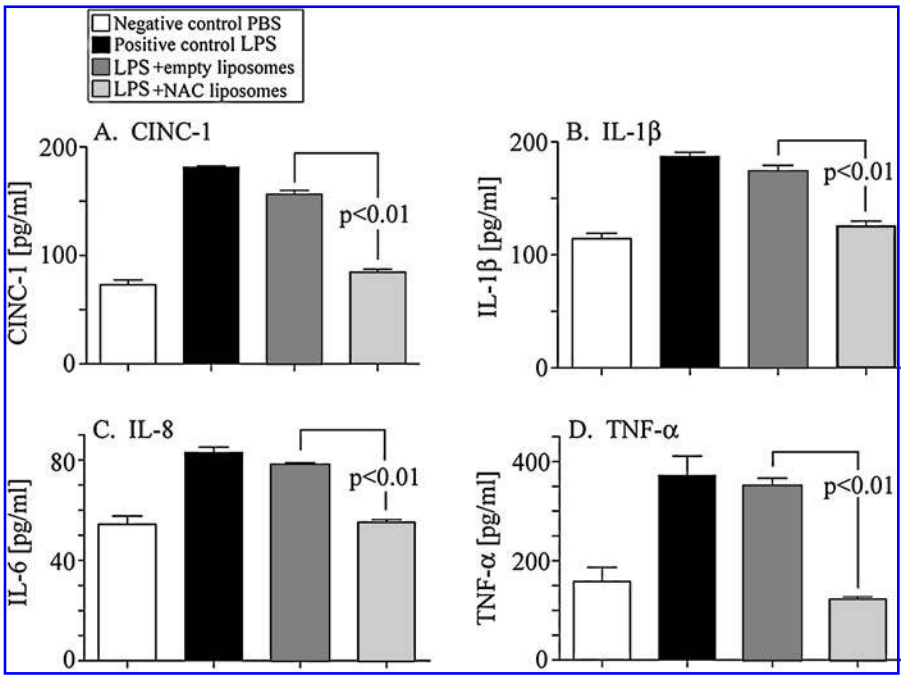

FIG. 4. Effects of LPS and liposomes on levels of proinflammatory mediators in 4-h culture fluids of alveolar macrophages (2.5×10^6) incubated with PBS (negative control) or with LPS (100 ng/ml) alone (positive control), or with empty liposomes or NAC-containing liposomes. Mediators CINC-1 (**A**), IL-1β (**B**), IL-6 (**C**), and TNF-α (**D**) were measured with ELISA. Each sample was measured in quadruplicate. For each bar, $n = 6$ animals.

used. In the first set of experiments, cells were exposed to LPS (100 ng/ml) for 4 hours in the absence or presence of empty or NAC-liposomes. Supernatant fluids were then measured for the presence of proinflammatory mediators, as shown in Fig. 4. In each case, LPS addition in the absence or presence of empty liposomes induced a substantial increase (over negative controls) of CINC-1, IL-1β, IL-6, and TNF-α. The presence of NAC-liposomes caused the mediator levels to decrease to baseline levels. Addition of empty liposomes did not induce mediator release from nonstimulated alveolar macrophages (data not shown).

In the second set of *in vitro* experiments (Fig. 5), the effect of CEES alone or in the presence of empty or NAC-containing liposomes on mediator release from alveolar macrophages was assessed. Empty liposomes did not perturb the levels of mediators released, remaining at baseline levels in nonstimulated

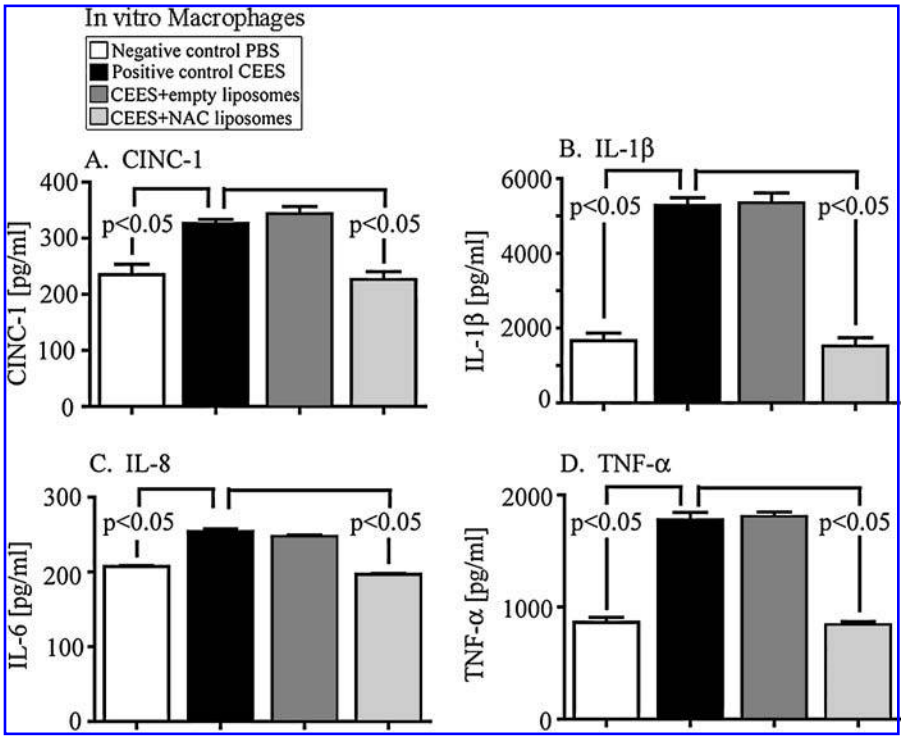

FIG. 5. *In vitro* effects of CEES and liposomes on levels of proinflammatory mediators in 4-h culture fluids of alveolar macrophages (2.5×10^6) incubated with PBS (negative control), CEES (500 μM) alone (positive control), or with empty liposomes or NAC-containing liposomes. Mediators CINC-1 (**A**), IL-1β (**B**), IL-6 (**C**), and TNF-α (**D**) were measured with ELISA. Each sample was measured in quadruplicate. For each bar, $n = 6$ animals.

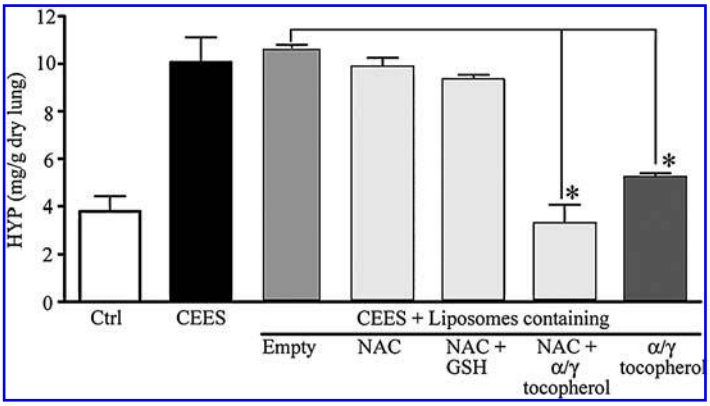

FIG. 6. Collagen levels (as measured by hydroxyproline content) in left lung homogenates 3 weeks after airway instillation of PBS (control) or CEES alone or together with empty liposomes or liposomes containing NAC, NAC + GSH, NAC + α, γ-tocopherol, or α, γ-tocopherol alone. For each bar, $n = 8$ animals. *$p < 0.05$ when compared with the CEES group.

macrophages (data not shown). Furthermore, in all cases, the addition of empty liposomes had no effect on mediator release when compared with CEES-stimulated macrophages not otherwise manipulated (Fig. 5). In contrast, the addition of NAC-containing liposomes to CEES-stimulated macrophages caused total suppression of the increase in levels of IL-6, IL-1β, TNF-α, and CINC-1 release, declining to levels equivalent to negative controls. Clearly, NAC-liposomes suppress release of proinflammatory mediators *in vitro* and *in vivo* after exposure of alveolar macrophages to either CEES (Fig. 5) or LPS (Fig. 4). The mechanisms by which CEES induces release of proinflammatory mediators from alveolar macrophages and the protective effects of NAC-liposomes are not known but may be linked to NF-κB activation or suppressed NF-κB activation.

Ability of anti-oxidant liposomes to protect from CEES-induced pulmonary fibrosis

We recently showed that CEES administration into rat lung induces intense pulmonary fibrosis within 3 weeks, as defined by histologic changes (29). In the current studies, we assessed whether antioxidant liposomes would prevent this outcome. Accordingly, CEES was administered into rat lungs in the absence of liposomes or together with empty liposomes or liposomes containing NAC, NAC + GSH, or NAC + α/γ-tocopherol or α/γ-tocopherol alone. Three weeks later, lungs were obtained, and collagen content (hydroxyproline, HYP) was assessed, as described earlier (31). As shown in Fig. 6, hydroxyproline content (mg/gm dry lung) increased 2.5-fold 3 weeks after CEES exposure of lungs in the absence of liposomes or in the presence of empty liposomes or liposomes containing NAC or NAC + GSH. No protective effects on hydroxyproline build-up were found. Strikingly, when liposomes containing NAC + α/γ-tocopherol were used, the build-up of hydroxyproline was completely abrogated ($p < 0.05$). Liposomes containing only α/γ-tocopherol also suppressed HYP buildup but not to the same extent as when α/γ-tocopherol liposomes were used ($p < 0.05$).

Morphologic correlates in lung

As shown in Fig. 7, sham lungs demonstrated normal architecture (A), whereas lungs exposed 3 weeks earlier to CEES showed extensive interstitial fibrosis (shown by blue staining in Masson-trichrome–stained sections), inflammatory cell infiltrates (neutrophils and mononuclear cells), and alveolar collapse (B). In contrast, CEES-exposed lungs from rats also given liposomes with NAC and α/γ-tocopherol had preservation of alveolar spaces and limited inflammatory cellular infiltrates, both in alveolar walls and in alveolar spaces (C). These data support the biochemical data shown in Fig. 6.

DISCUSSION

These studies establish that antioxidant liposomes can protect against CEES-induced ALI in rats after airway instillation of appropriate liposomes. ALI, as defined by breakdown of the vascular endothelial and alveolar epithelial barriers with leak of ^{125}I-albumin into lung parenchyma, occurred in rats after airway exposure to CEES. In the setting of CEES, development of ALI is related to complement activation, PMN influx, generation predominantly by activated lung macrophages of proinflammatory cytokines and chemokines, and release of oxidants and proteases from PMNs and macrophages (9, 50). In other words, ALI caused by exposure to CEES is fundamentally caused by triggering of an extremely robust intrapulmonary inflammatory response, which is complement dependent (5). The vascular (endothelial) and airway (alveolar epithelial) barriers are breached by this intense inflammatory response, resulting in alveolar flooding and hemorrhage. It is possible that interaction of CEES with endothelial and epithelial cells also directly results in tissue damage (32). Some of the mechanisms of CEES-induced cell injury have recently been established. CEES is an alkylating agent and has variably toxic, mutagenic, carcinogenic, and teratogenic effects. However, the mechanisms of its delayed toxic effects (retardation in the rate of cell division, disruption of mitoses, chromosomal breakages, and other abnormalities of chromosomes) and of its carcinogenic actions are not understood (5). A review of the evidence linking sulfur mustard to oxidative stress has been described (32). Direct evidence for free radical formation in rat lung lavage after inhalation of sulfur mustard vapor has been obtained by using electron paramagnetic resonance (EPR) and spin-trapping techniques (1). These studies show a rapid formation of ascorbyl radicals followed by the formation of carbon-centered radicals. Evidence for free radical–mediated lung damage in CEES-exposed mice (*i.e.*, increased lipid peroxidation, as well as decreased GSH and increased oxidized GSH) has also been described) (17). Interestingly, it has been shown that CEES is

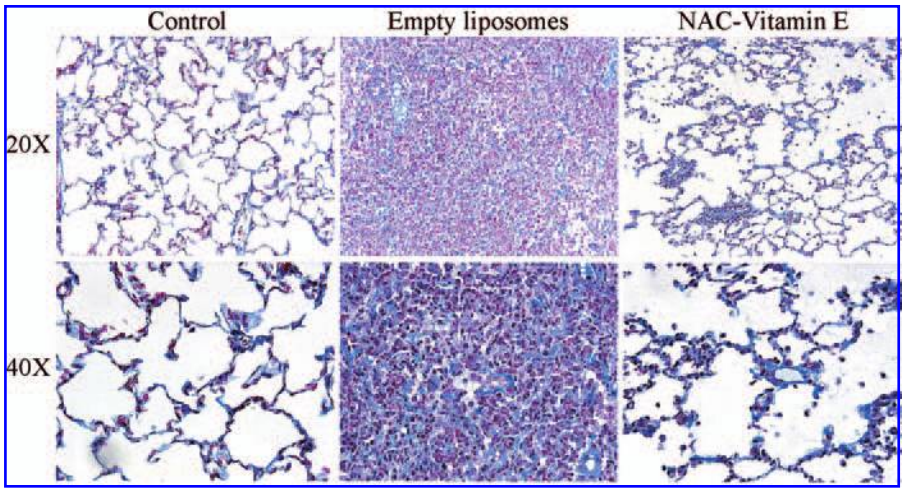

FIG. 7. Histologic features of control lung (airway instillation of PBS), CEES lung with empty liposomes, or CEES lung with liposomes containing NAC + α/γ-tocopherol (vitamin E). Lungs were obtained 3 weeks after airway instillation of CEES. (Masson Trichome staining of paraffin-embedded sections, 20× and 40× magnification).

an alkylating agent that will react with nonprotein thiols (NAC or GSH) as well as protein thiol groups. Mustard gas is known to form metabolites (by the β-lyase pathway) derived from its covalent conjugation with GSH. Sulfur mustard metabolites from the glutathione (GSH)/β-lyase pathway are specific markers for exposure to sulfur mustard (6, 7). This may represent another mechanism by which antioxidant liposomes protect against CEES-induced lung injury.

In the setting of our lung-injury model, all of these changes result in acute lung injury (4 h), followed by alveolar collapse and interstitial pulmonary fibrosis 3 weeks later. These outcomes can be averted by liposomes that contain the combination of NAC and α/γ-tocopherol or α/γ-tocopherol alone, whereas liposomes containing α/γ-tocopherol did not attenuate the acute lung injury. In contrast, liposomes containing NAC or NAC + GSH did not attenuate long-term fibrosis (3 weeks) but proved to be efficacious in protecting against CEES-induced ALI (4 h). Why liposomes containing certain antioxidants or combinations thereof are effective in the acute injury but not in the long-term injury, and *vice versa*, is not clear. It seems that tocopherol and NAC affect different aspects of the acute reaction, whereas tocopherol is more effective in preventing the progression to fibrosis. One possible explanation might be the hydrophobic nature of α/γ-tocopherol, which allows insertion into membranes (cell membrane, endoplasmatic reticulum, and mitochondrial membranes), producing a relatively long-lasting effect, whereas internalization of NAC might lead to a relatively short life span (48). Similar to our results, it has been reported that the instillation of liposomes containing both GSH and α-tocopherol (but not GSH alone) resulted in the highest level of GSH retention in lung tissue, suggesting an effective antioxidant formulation for treating oxidative lung injury (43). In addition, it has been shown that tocopherol may also modulate signal-transduction pathways (2, 35, 52). Precisely what products of the macrophage can be linked to the development of pulmonary fibrosis after lung exposure to CEES remains to be defined, but a likely candidate mediator known to be released by macrophages is TGF-β (4). The protective effects of NAC-liposomes after acute exposure of lung to CEES liposomes may be associated with the dramatic disappearance of proinflammatory mediators in BAL fluids appearing after exposure to CEES (Fig. 3). Suppression of mediator appearance may be related to the ability of intracellular NAC to suppress NF-κB activation within macrophages, preventing generation of proinflammatory mediators. However, it should be noted that we did not directly measure a "restoration of redox balance," but other investigators, by using *in vitro* model systems, have shown that NAC protects against CEES toxicity by blocking the CEES-induced loss of intracellular GSH (22). We therefore assume that the administration of antioxidant liposomes affects the redox status in lung cells. It remains to be shown in future experiments whether antioxidant agents indeed restore the redox balance within the different lung cells.

Liposomal use for drug delivery has been studied extensively [reviewed in (13)]. The main cellular targets for lung oxidant stress are epithelial cells lining the alveoli (including type I and II alveolar cells), endothelial cells lining the pulmonary capillaries, and alveolar macrophages. Liposomal drugs may be administered orally, intravenously, or intratracheally, but only the latter two routes improve drug delivery (13). Intravenously administered liposomal drugs may be absorbed by pulmonary endothelial cells, but providing effective methods of drug delivery is still challenging.

For intratracheal instillation, the uptake and subcellular distribution of liposomal α-tocopherol (but not γ-tocopherol) in lung tissue was examined (41). Recovery of α-tocopherol in the lung was maximal 1 hour after liposomal instillation and resulted in a 16-fold increase in pulmonary total α-tocopherol concentration 72 h after instillation. α-Tocopherol was recovered largely from cytosolic, nuclear, microsomal, and mitochondrial fractions, providing evidence that α-tocopherol levels present in the membranes of these subcellular fractions were sufficient to protect against oxidant-induced lipid peroxidation. Evidence suggests that liposomes given into the airways are avidly taken up by phagocytes of the reticuloendothelial system (21, 27, 39, 46). Once internalized, they release their contents intracellularly (34, 36). In addition, alveolar types I and II as well as pulmonary endothelial cells have been shown to be able to internalize intratracheally administered liposomes (3, 33). In the current studies, we focused on alveolar macrophages (which are the chief sources of lung cytokines and chemokines) being affected by NAC-liposomes (Fig. 3); however, to what extent other lung cells such as alveolar epithelial cells have been altered remains to be determined.

The implications from the current studies (especially *in vitro* data) are that liposomal delivery most likely enhances a reduc-

ing environment in lung macrophages, which may be otherwise compromised when these cells come into contact with CEES or LPS. Delivery of NAC in liposomes may be protective in the setting of CEES-induced lung injury by phagocytosis of liposomes into lung macrophages and possibly by other lung cells as well (21, 27, 39). Once ingested by macrophages, the liposomal contents are released into the interior of the macrophages (34, 36). NAC may be distributed into the cytosol component, whereas α/γ-tocopherol is distributed into membranes within cells (see earlier). Phosphonate-containing liposomes have been used in rodents selectively to deplete the lung of macrophages, whereas other types of lung cells are not affected (49). It seems in the CEES model of ALI that the release of proinflammatory mediators (cytokines and chemokines) from lung macrophages is blocked by the use of NAC-liposomes, subsequently resulting in intracellular release of NAC and blockade of NF-κB activation within macrophages. However, it seems plausible that an attenuated oxidative stress within macrophages may also indirectly result in protection of adjacent alveolar epithelial and endothelial cells. Whether the use of other liposomes with antioxidant properties (*e.g.*, GSH, α/γ-tocopherol) would also suppress the release into CEES-treated lungs of proinflammatory mediators remains to be determined, but it seems likely, because such liposomes protect against ALI after airway exposure (44). As suggested earlier, it is likely that NAC liposomes are phagocytized by lung macrophages, resulting in release of NAC into the cytosol, resulting in blockade of NF-κB activation, which is required for production and release of mediators by macrophages. The trigger in this situation is likely CEES, which causes NF-κB activation in macrophage-type cells (9, 14).

Lipopolysaccharide (LPS) is known to induce acute lung injury with features similar to changes in CEES-induced acute lung injury (50). Because both types of injury result in an inflammatory response and its accompanying oxidative stress, we extended our experiments to *in vitro* exposure of alveolar macrophages to LPS or CEES (Figs. 3 and 4). *In vivo* experiments have shown that administration of liposomes containing NAC or α-tocopherol protects rats from ALI induced by LPS or paraquat (19, 42, 44, 45). We show in this report that liposomes containing NAC significantly suppressed the release of proinflammatory cytokines from alveolar macrophages exposed to either LPS or CEES, suggesting similar pathways being activated under both conditions. This confirms the *in vivo* data (Fig. 3), underscoring the hypothesis that augmentation of the pulmonary antioxidant status can attenuate both LPS- and CEES-induced oxidative stress.

Taken together, restoration of the redox balance may be crucial in the setting of ALI induced by CEES for attenuation of early, acute injury, and may also set the stage for attenuating the long-term effects of CEES (pulmonary fibrosis). Liposome-mediated delivery of antioxidant agents, particularly NAC and α/γ-tocopherol, is a powerful tool to diminish CEES-induced acute and long-term lung injury.

ABBREVIATIONS

ALI, acute lung injury; ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; BSA, bovine serum albumin; ^{125}I-BSA, iodine 125–labeled bovine serum albumin; CINC-1, cytokine-induced neutrophil chemoattractant-1; CEES, chloroethyl ethyl sulfide; DPBS, Dulbecco's phosphate-buffered saline; DPPC, dipalmitoylphosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; GSH, glutathione; HYP, hydroxyproline; IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κ-B; PMN, polymorphonuclear leukocyte; RPMI, Roswell Park Memorial Institute; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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