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Alleviation of Paraquat-Induced Lung Injury by Pretreatment with Bifunctional Liposomes Containing α-Tocopherol and Glutathione

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ABSTRACT. Reactive oxygen species are known to play a key role in the development of acute lung injury, and such injury can be alleviated by pretreating the lung with a suitable antioxidant preparation. In this study, we evaluated and compared the antioxidant efficacy of two liposomal preparations: liposomes containing only α-tocopherol versus bifunctional liposomes containing both α-tocopherol and glutathione (GSH). α-Tocopherol liposomes (2 mg α-tocopherol/animal) or liposomes containing both α-tocopherol and GSH (2 mg α-tocopherol and 10 μmol GSH/animal) were intratracheally instilled into the lungs of rats 30 min prior to a challenge with paraquat dichloride (30 mg/kg, i.p.); animals were killed 24 hr post-paraquat challenge. Lungs of paraquat-challenged animals were damaged extensively as evidenced by increases in lung weight, indicative of edema, and decreases in lung activities of angiotensin converting enzyme (ACE) and alkaline phosphatase (AKP), indicative of endothelial and alveolar type II epithelial cell injuries, respectively. While the pretreatment of rats with α-tocopherol liposomes or liposomes containing both α-tocopherol and GSH significantly attenuated paraquat-induced changes in lung ACE activity to more or less the same extent, the bifunctional liposomal preparation conferred additional protection to alveolar type II epithelial cells, as evidenced by a significantly higher pulmonary AKP activity. Our results also showed that both liposomal preparations failed to ameliorate paraquat-induced lung edema despite a significant protection of pulmonary endothelial cells, suggesting that paraquat-induced edema formation may be independent of endothelial cell damage. In conclusion, liposome-associated antioxidants can protect the lung against an oxidant challenge, and the extent of protection appears to be related to the characteristics of each antioxidant formulation. BIOCHEM PHARMACOL 52;10:1515–1520, 1996. *Published 1996 Elsevier Science Inc.*

KEY WORDS. paraquat; lung injury; bifunctional liposomes; glutathione; α-tocopherol; oxidative stress

Exposure of humans and experimental animals to paraquat dichloride, a broad-spectrum herbicide, results in lung injury manifested by edema, haemorrhage, interstitial inflammation, and proliferation of bronchial epithelium [1–4]. These manifestations are similar to those observed in the adult respiratory distress syndrome associated with sepsis, burns, and exposure to certain drugs and pollutants [5–8]. The similarities among the different models of acute lung injury have been attributed largely to a common mechanistic event, namely oxidative stress [1–8].

Recognizing that paraquat injures the lung primarily via oxidative stress-mediated mechanisms [1–4], we have attempted to develop liposome-based pharmacological strategies to counteract paraquat-induced tissue injuries by reducing the formation of reactive oxygen species and/or preventing their toxic effects. Thus, the instillation of liposome-associated α-tocopherol directly into the lung in rats has resulted in a significant increase in the pulmonary α-tocopherol concentration, and such treatment has also been shown to reduce significantly the toxic effects of the herbicide [9, 10]. α-Tocopherol, the main constituent of vitamin E, is known to reduce lipid peroxidation by functioning as a free radical scavenger and by quenching singlet molecular oxygen, thus contributing to cell membrane stabilization [11–13]. α-Tocopherol, however, is a highly insoluble and viscous substance, not amenable to direct administration to the lung. The incorporation of α-tocopherol in liposomes renders it easy for instillation into the respiratory system, thus promoting its pharmacological potential. Liposomes are also known to aid in the transfer of macromolecules, normally impermeable to cell membranes, to the cell interior, and liposome entrapment also prolongs the half-lives of these macromolecules [9, 10, 14–17].

Liposomes are essentially phospholipid vesicles consisting of one or more lipid bilayers enclosing an aqueous space. Lipophilic agents can be incorporated in the lipid bilayer

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while hydrophilic agents can be encapsulated in the aqueous space of the liposomes. We took advantage of the amphipathic nature of liposomes by preparing a bifunctional formulation that incorporates in the same liposomal carrier, α-tocopherol (a lipophilic antioxidant) in the lipid bilayers and GSH§ (a hydrophilic antioxidant) in the aqueous compartment. The concept of bifunctional liposomes is to enable the simultaneous delivery of two entrapped agents that possess different mechanisms of action, thereby increasing the potential of a better treatment effect [18].

We have shown previously that liposomal α-tocopherol can protect the lung against paraquat-induced lung damage [9]. In this study, we examined whether the antioxidant efficacy of α-tocopherol-containing liposomes can be augmented by the co-entrapment of a water-soluble antioxidant such as GSH. α-Tocopherol acts as an antioxidant by scavenging reactive oxygen species, by quenching singlet molecular oxygen, and by modifying the physicochemical properties of biological membranes [11–13]. GSH serves as a reductant in the metabolism of hydrogen peroxide and various organic hydroperoxides, a reaction catalyzed by GSH peroxidases present in the cytosol and mitochondria of various cells, and it can also regenerate α-tocopherol from its oxidation products [19–23]. Thus, our hypothesis is that a combination of two antioxidants, known to exert their antioxidant effects via different pathways, should provide better protection against paraquat-induced pulmonary toxicity. Lung injury was assessed by determining changes in total lung weight and also changes in pulmonary levels of specific enzyme markers, ACE and AKP, indicative of injuries to pulmonary endothelial cells and alveolar type II epithelial cells, respectively.

MATERIALS AND METHODS

Chemicals

Paraquat dichloride, GSH, and α-tocopherol were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). All other chemicals were obtained from Sigma or BDH (Toronto, Ontario, Canada).

Animals

Male Sprague–Dawley rats (approximate body weight 220–250 g) were purchased from Charles River Canada, Inc. (St. Constant, Quebec, Canada). All animals were housed in stainless-steel cages with free access to pelleted Purina laboratory chow and tap water. The animals were kept at room temperature (22–24°) and were exposed to alternate cycles of 12 hr light and darkness. Animals used in this study were treated and cared for in accordance with the guidelines contained in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care.

Preparation of Liposomes Containing α-Tocopherol and/or GSH

The procedure for the preparation of α-tocopherol DPPC liposomes and the entrapment of GSH in α-tocopherol liposomes was performed as previously described [10]. Liposomal vesicle size was determined with the use of a Coulter N4SD particle-size analyzer and was found to have a mean diameter of 370 ± 58 nm.

Treatment of Animals

α-Tocopherol liposomes (2 mg α-tocopherol/animal) or α-tocopherol liposomes containing GSH (10 μmol GSH/animal) were intratracheally instilled into the lungs of rats as previously described [10]. Thirty minutes after administration of the liposomal formulations, rats were injected i.p. with a single dose (30 mg/kg) of paraquat dichloride (LD_{50}: 35 mg/kg) to induce pulmonary toxicity. Injections were administered between 8:00 and 9:00 a.m. Paraquat dichloride was dissolved in saline and prepared shortly before use. Control animals received an equivalent volume of the vehicle solution.

Experimental Design

To investigate whether the direct delivery of α-tocopherol liposomes containing GSH to the lung would improve the antioxidant efficacy of α-tocopherol liposomes, rats pretreated with the antioxidant formulations were challenged with a single dose of paraquat dichloride and killed 24 hr later. The protective effect of the antioxidant formulations against paraquat-induced lung damage was assessed biochemically by measuring the activities of ACE and AKP as well as the content of GSH in the lung.

Tissue Preparation

Lungs were removed from animals immediately after decapitation and rinsed with ice-cold saline to remove excess blood. All subsequent steps were carried out at 0–4°. Following rinsing, lungs were quickly weighed and finely minced. Approximately 1 g of lung sample was homogenized with a Brinkmann Polytron in a sufficient volume of ice-cold 50 mM potassium phosphate buffer, pH 7.4, to produce a 20% homogenate.

Enzyme Measurements

The activity of ACE was determined using the Sigma Diagnostic procedure as previously described [10]. One unit of ACE activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol furylacryloylphenylala-

§ *Abbreviations:* ACE, angiotensin converting enzyme; AKP, alkaline phosphatase; DPPC, dipalmitoylphosphatidylcholine; and GSH, glutathione.

nine/min at 37°. AKP activity was determined as previously described [10], and one unit of AKP activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol *p*-nitrophenol/min at 37°. Protein determinations were estimated by the method of Lowry *et al.* [24], using crystalline bovine serum albumin as the standard.

Determination of Tissue GSH Contents

GSH contents, more precisely non-protein sulfhydryl, in pulmonary homogenates were determined as described by Suntres and Lui [25]. Briefly, the tissue was homogenized in 20% (w/v) trichloroacetic acid and centrifuged at 9000 *g* for 20 min in a refrigerated Sorval RC-5B centrifuge. An aliquot of the supernatant fraction in 0.3 M phosphate buffer was treated with 5,5-dithiobis-[2-nitrobenzoic acid], and the absorbance at 412 nm was measured.

Statistical Analysis

Data from different groups of animals pretreated with saline, α-tocopherol liposomes, and α-tocopherol/GSH liposomes were evaluated by two-way ANOVA [26]. The level of significance was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

A considerable number of antioxidants have been utilized either prophylactically or therapeutically in an attempt to prevent or ameliorate lung injuries induced via oxidative stress-mediated mechanisms [9, 10, 15, 16]. It has been demonstrated that pretreatment of animals with superoxide dismutase and catalase provides limited protection against pulmonary oxidants because of their inability to cross biological membranes [9, 10, 14–17]. Similarly, administration of GSH has been shown to be ineffective in the treatment of oxidant-induced lung injury due to its rapid clearance from the lung [17, 27, 28]. Moreover, the extreme insolubility of some antioxidants such as α-tocopherol have made these antioxidants unsuitable for use in emergency situations [29, 30]. All these limitations, however, have been circumvented by incorporating antioxidants into liposomes. Incorporation of antioxidants into liposomes provides a formulation that enhances drug uptake, delays rapid drug clearance, and reduces drug toxicity [9, 10, 14–17, 29, 30].

Enzyme Markers of Lung Injury

Results from this and previous studies have revealed that pretreatment of animals with liposome-associated α-tocopherol ameliorated paraquat-induced lung injuries [9, 10]. Since ACE, an enzyme primarily localized in pulmonary capillary endothelial cells, has been used as a marker of lung injury [31], the effects of paraquat dichloride on the activity of this enzyme in the lung tissue of animals pretreated with saline or liposomes containing antioxidants were measured in this study. As shown in Fig. 1A, challenge of saline-pretreated animals with paraquat dichloride resulted in a significant decrease in pulmonary ACE activity. Animals pretreated with liposomes containing α-tocopherol or α-tocopherol and GSH suffered a significantly less reduction in pulmonary ACE activity after paraquat challenge in comparison to that of animals in the saline-control group. It has been shown that α-tocopherol associated with liposomes or liposomal fragments can be transferred to cellular membranes quite effectively, a treatment effect resulting in high concentrations of the lipophilic antioxidant in cell membranes [32, 33]. Administration of GSH encapsulated in α-tocopherol liposomes was expected to confer an increased protection against paraquat-induced endothelial cell damage, but contrary to expectations no additional

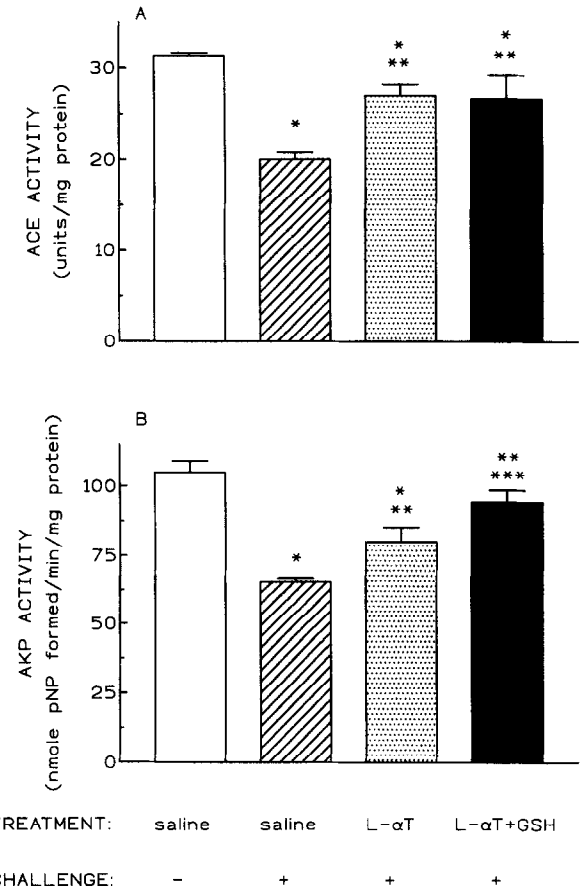

FIG. 1. Effect of pretreatment with liposomes containing α-tocopherol (L-αT) or α-tocopherol and GSH (L-αT + GSH) on paraquat-induced changes in ACE and AKP activities. Animals were pretreated, as indicated and described in Materials and Methods, 30 min prior to a challenge with paraquat dichloride (PQ, 30 mg/kg, i.p.); then they were killed 24 hr later, and their lungs were obtained for the determination of ACE and AKP activities. Each data point is the mean ± SEM of 5 animals. Key: (*) significantly different ($P < 0.05$) from the value of control animals pretreated with saline but not challenged with PQ; (**) significantly different ($P < 0.05$) from the value of animals pretreated with saline and challenged with PQ; and (***) significantly different ($P < 0.05$) from the value of animals pretreated with α-tocopherol liposomes and challenged with PQ.

protection was observed (Fig. 1A). This lack of additional endothelial protection occurred despite the restoration of the GSH content in the lung to above normal level (Table 1). Thus, the residual endothelial damage must have occurred via mechanisms independent of GSH depletion.

AKP activity has been shown to be localized primarily in alveolar type II epithelial cells and has been used as a marker of lung injury as well [34]. Exposure of saline-pretreated animals to paraquat dichloride resulted in alveolar type II epithelial cell injury, as indicated by a significant decrease in pulmonary AKP activity (Fig. 1B). Pretreatment of animals with α-tocopherol liposomes resulted in a significant protection against paraquat-induced changes in AKP activity. Moreover, the paraquat-induced decrease in AKP activity was even less evident in the lungs of rats pretreated with α-tocopherol liposomes also containing GSH, suggesting that GSH encapsulated in α-tocopherol liposomes improves the antioxidant efficacy of the α-tocopherol formulation. The specific toxicity of paraquat for lung tissue has been explained by its selective accumulation in alveolar type I and II epithelial cells and Clara cells [1–4, 35]. Injury to alveolar type II epithelial cells results in marked lung dysfunction due to failure of these cells to produce surfactant, a material that reduces surface tension in the lung, and their inability to proliferate to alveolar type I epithelial cells,which play a critical role in preserving the functional integrity of the alveolar surface.

The mechanism of the additional protective effect of α-tocopherol liposomes containing GSH against paraquat-induced alveolar type II epithelial cell damage cannot be delineated from the results of this study. Results from studies examining the distribution of intratracheally administered liposomal agents have indicated that these preparations are taken up predominately by alveolar type II epithelial cells as well as macrophages and cells localized in the alveolar septae [36, 37]. Moreover, it has been demonstrated that these liposomal formulations, once taken up by alveolar type II cells and macrophages, are subject to degradative processes leading to the release of the encapsulated agent, and subsequently to an increase in the concentration of the free agent at the site of action [36–39]. In light of these observations, it is conceivable that the additional protective effects of the liposomal formulation containing both antioxidants may be due, at least in part, to an increase in the localization of free glutathione in alveolar type II cells.

TABLE 1. Pulmonary levels of GSH in rats

Pretreatment	Paraquat challenge	GSH content (μmol/lung)
Saline	−	1.90 ± 0.10
Saline	+	1.47 ± 0.13*
α-Tocopherol liposomes	+	1.67 ± 0.08*†
α-Tocopherol/GSH liposomes	−	4.05 ± 0.28*
α-Tocopherol/GSH liposomes	+	2.40 ± 0.37*‡

Animals were pretreated, as indicated and described in Materials and Methods, 30 min prior to challenge with an i.p. injection of paraquat (30 mg/kg body wt) and were killed 24 hr later; the GSH content of each lung was determined. Each value is the mean ± SEM of 5 animals.

* Significantly different ($P < 0.05$) from the value of animals pretreated with saline, but not challenged with paraquat.

† Significantly different ($P < 0.05$) from the value of saline-pretreated animals challenged with paraquat.

‡ Significantly different ($P < 0.05$) from the value of animals pretreated with α-tocopherol/GSH liposomes, but not challenged with paraquat.

Delivery of GSH and α-Tocopherol to the Lung

Since GSH is known to play an important role in protecting cells from oxidant-induced tissue injury [40, 41], GSH levels in the lungs of saline-pretreated and liposome-pretreated animals were also measured. Results presented in Table 1 reveal that GSH contents in the lungs of saline-pretreated rats exposed to paraquat dichloride were decreased significantly 24 hr post-paraquat treatment. Similarly, a significant paraquat-induced decrease in the GSH content was also observed in lungs of rats pretreated with α-tocopherol liposomes, but levels were significantly higher than those observed in lungs of saline-pretreated rats exposed to paraquat dichloride. Pretreatment of animals with α-tocopherol liposomes containing GSH resulted in significant increases in pulmonary GSH content by about 2-fold (Table 1). Paraquat treatment of this liposomal pretreated group resulted in significant reductions in the pulmonary GSH content of rats (by approximately 40%) with the remaining pulmonary GSH levels being higher than those observed in the other groups.

It is generally accepted that a significant elevation of α-tocopherol or GSH in the lung is difficult to achieve if these antioxidants are administered via non-tracheal routes. For example, parenteral or oral administration of α-tocopherol in animals resulted in the recovery of only 36 or 13 μg/g lung, respectively [42, 43]. Exogenously delivered GSH is hydrolyzed rapidly in the kidneys into its constituent amino acids, which are then redistributed and resynthesized to the tripeptide, primarily in the liver [44, 45]. Under our experimental conditions, intratracheal administration of α-tocopherol liposomes or α-tocopherol liposomes containing GSH results in a substantial increase in pulmonary total α-tocopherol [29] and GSH levels [17, 46]. It is apparent from the results of this study that liposome-mediated antioxidant delivery achieves the attainment of sufficient antioxidant levels in the respiratory milieu to alleviate paraquat-induced oxidative damages.

Wet Lung Weight

Both *in vivo* and *in vitro* studies have shown that paraquat can injure alveolar type II epithelial cells and capillary endothelial cells. Injury to the air-blood barrier and impairment of surfactant production in the lung can cause pulmonary edema and collapse of the fine airways. The effect of intraperitoneally administered paraquat dichloride on lung weight of animals pretreated with saline, α-tocopherol liposomes, or liposomes containing both α-tocopherol and

TABLE 2. Effect of antioxidant pretreatment on paraquat-induced changes in lung weight

Pretreatment	Paraquat challenge	Wet lung weight (g)
Saline	−	1.18 ± 0.04
Saline	+	1.50 ± 0.10*
α-Tocopherol liposomes	+	1.61 ± 0.15*
α-Tocopherol/GSH liposomes	−	1.22 ± 0.05
α-Tocopherol/GSH liposomes	+	1.65 ± 0.11*

Animals were pretreated, as indicated and described in Materials and Methods, 30 min prior to challenge with an i.p. injection of paraquat (30 mg/kg body wt) and were killed 24 hr later; the wet weight of each lung was determined. Each value is the mean ± SEM of 5 animals.

* Significantly different ($P < 0.05$) from the value of control animals pretreated with saline, but without paraquat challenge.

GSH is shown in Table 2. Lung weights of saline-pretreated animals were increased significantly by 24 hr post-paraquat challenge. Pretreatment of animals with α-tocopherol liposomes or liposomes containing both α-tocopherol and GSH did not alter significantly the paraquat-induced changes in lung weight. Thus, the administration of antioxidants failed to modify paraquat-induced edema. The lack of protective effect by the antioxidants against paraquat-induced edema cannot be ascertained at the present time, but results from other studies have demonstrated that the edema may be due to mechanisms other than direct injury of pulmonary endothelial cells. This suggestion corroborates the results of this study where pulmonary edema persisted despite an apparent reduction in endothelial and alveolar type II epithelial cell injury.

Conclusion

Based on results of the present study, it can be concluded that the administration of liposomes containing more than one antioxidant is advantageous in ameliorating paraquat-induced lung injury. The demonstrated efficacy of our bifunctional antioxidant liposome formulation also implicates its possible application as a potential prophylactic agent in alleviating oxidant-induced tissue injuries in procedures such as hemodialysis, surgical operations, and blood reperfusion.

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