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The Pulmonary Uptake of Intravenously Administered Liposomal α-Tocopherol is Augmented in Acute Lung Injury

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The present study was carried out to investigate whether the intravenous administration of liposomal α-tocopherol can result in a significant localization of the antioxidant in the injured lung. Male Sprague-Dawley rats were injected with paraquat dichloride (20 mg/kg, ip.) and 4, 24 or 48 h later, they were given an intravenous injection of a liposomal α-tocopherol preparation (20 mg α-tocopherol in 128 μmoles liposomal lipid/kg) labelled with [^{14}C]dipalmitoylphosphatidylcholine (DPPC) and [^{3}H]α-tocopherol. Animals were killed and their lungs removed for analysis 24 h after liposomal treatment. To demonstrate whether the extent of uptake of radioactive α-tocopherol liposomes was directly related to the extent of residual lung injury, additional groups of animals were also injected with higher doses (30 and 40 mg/kg body weight) of paraquat dichloride and 48 h later, were treated with liposomal α-tocopherol; animals were then killed 24 h after liposomal α-tocopherol treatment. The intraperitoneal injection of paraquat dichloride resulted in time- and dose-dependent decreases in angiotensin converting enzyme and alkaline phosphatase activities suggesting that the toxicant injures both the capillary endothelial cells and alveolar type II epithelial cells, respectively. The recovery of intravenously administered radioactive α-tocopherol in the lungs of saline-treated animals was found to be about 2% of the initial dose 24 h post-liposomal treatment. However, in paraquat-treated animals, there was an increased localization of the labelled α-tocopherol to the lung, resulting in a difference of pulmonary delivery by as much as 2–3 fold compared to that in a normal lung. The ^{3}H/^{14}C ratio, representing the recovery of [^{3}H]α-tocopherol and [^{14}C]liposomes, was practically constant and there was a linear relationship between the measurable lung injury index and the corresponding recovery of radiolabelled α-tocopherol in the lung. Our results appear to suggest that the residual pulmonary injury augments the delivery of liposomal α-tocopherol to the lung.

Keywords: liposome, antioxidant, α-tocopherol, lung injury, drug delivery

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INTRODUCTION

Acute lung injury is one of the major causes of morbidity and mortality in clinical conditions associated with sepsis, trauma, burns and exposure to certain xenobiotics and pollutants. Acute lung injury may be characterized by an increase in alveolar-capillary permeability, edema, severe hypoxemia, fibrosis and other physiological and biochemical abnormalities (Blennerhassett, 1985; Kehrer and Kacew, 1985; Villar and Slutsky, 1989; McLean and Byrick, 1993; Muller and Herndon, 1994).

Reactive oxygen species play a key role in the sequence of events leading to the development of acute lung injury (Kehrer and Kacew, 1985; McLean and Byrick, 1993; Cross et al., 1994). In general, reactive oxygen species cause injury by disrupting critical cell functions and by activating the inflammatory response, resulting in the recruitment and activation of phagocytic cells to generate additional toxic reactive oxygen species (Boobis et al., 1989; Janssen et al., 1993; Cross et al., 1994).

So far, therapeutic measures for treating oxidant-mediated acute lung injury have been mainly supportive due to a lack of pharmacological agents efficacious enough to ameliorate the oxidant-induced lung injury (Byrne et al., 1987; Weinberger, 1993). One of the strategies for modifying oxidant-mediated lung injuries is to increase the antioxidant capacity of cells by administering exogenous superoxide dismutase, catalase, glutathione and deferoxamine (Turrens et al., 1984; Padmanabhan et al., 1985; Panus and Freeman, 1988; Ward et al., 1988; Suntres et al., 1992; Schiller et al., 1993; Shek et al., 1994). Such approach, however, has had only limited success because these antioxidants have relatively short half-lives and are usually unable to gain access to the cell interior in a sufficiently large concentration to increase the cellular antioxidant content. The use of liposomes as carriers for the delivery of antioxidants to the lung has been attempted and the results have been encouraging (Turrens et al., 1984; Padmanabhan et al., 1985; Panus and Freeman, 1988; Ward et al., 1988; Suntres et al., 1992; Shek et al., 1994).

Liposome-entrapped antioxidants have been shown to be relatively effective in treating oxidant-induced lung injuries, either as a prophylactic or therapeutic measure (Turrens et al., 1984; Padmanabhan et al., 1985; Panus and Freeman, 1988; Suntres et al., 1992; Shek et al., 1994; Suntres and Shek, 1995). The pulmonary deposition of administered liposomal agents appears to depend on at least two factors: the route of administration (e.g., intratracheal vs intravenous) and the timing of the administration before or after (prophylactic vs therapeutic) the induction of lung injury. While the administration of a liposomal agent via the intratracheal route has the distinct advantage of delivering a large effective dose to the lung within a relatively short time, the intravenous route is also useful because it provides a simple and convenient way for drug administration to critical care patients with respiratory disease complications.

In studies where liposome-entrapped antioxidants were administered intravenously, only small amounts of the liposomal drug were found to reach the normal lung with most of the drug accumulating in the liver and spleen (Poste et al., 1984; Senior, 1987). There have been little or no studies examining the pulmonary uptake of intravenously administered liposomes by the injured lung. α-Tocopherol, one of the most potent hydrophobic antioxidants, is known to exert it antioxidant effects by scavenging reactive oxygen species and by reducing membrane lipid peroxidation. Although the extremely high insolubility of α-tocopherol renders it very difficult, if not impossible for intravenous injection, this lipophilic antioxidant can be readily entrapped in liposomes for non-parenteral administration (Suntres and Shek, 1995). Thus, the present study was designed to examine the deposition of an intravenously injected liposomal α-tocopherol preparation in normal and oxidant-injured lungs.

MATERIALS AND METHODS

Chemicals

Paraquat dichloride and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) was obtained from

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Avanti Polar Lipids (Alabaster, AL). [^{3}H]α-tocopherol and [^{14}C]DPPC were purchased from New England Nuclear, DuPont, Canada (Mississauga, Ontario). All other chemicals were obtained from either Sigma Chemical Co. (St. Louis, MO) or BDH (Toronto, Ont.).

Animals

Male Sprague-Dawley rats (approximate body weight 220–250 g) were purchased from Charles River Canada, Inc. (St. Constant, Que.). 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°C) and were exposed to alternate cycles of 12 h light and darkness. Animals used in this study were treated and cared for in accordance with the guidelines recommended by the Canadian Council on Animal Care in the *Guide to the Care and Use of Experimental Animals*.

Preparation of α-Tocopherol Liposomes

α-Tocopherol liposomes were prepared from DPPC and α-tocopherol in a 7:3 molar ratio and the dried lipid film was hydrated at 50°C in 5 mM phosphate buffer, pH 6.5, containing 3mM EDTA as described by Suntres et al. (1992). The liposomal preparation was labelled with [^{14}C]DPPC (specific activity: 2.5 mCi/mmol) and [^{3}H]α-tocopherol (specific activity: 5.0 mCi/mmol). Liposomal suspensions were extruded (10-times) with an extruder (Lipex Biomolecules, Vancouver, BC), fitted with two stacked polycarbonate filters of 400 nm pore size, using a helium pressure of 100–200 psi. Liposomal vesicle size was determined with the use of a Coulter N4SD particle-size analyser and was found to have a mean diameter of 311 ± 24 nm.

Treatment of Animals

Paraquat dichloride (20, 30 or 40 mg/kg body weight) was injected intraperitoneally in rats. Four, 24, or 48 h after administration of paraquat dichloride, rats were injected intravenously via the tail vein with α-tocopherol liposomes (20 mg α-tocopherol in 128 μmoles liposomal lipid/kg). Injections were carried out between 0800–0900 h. Paraquat dichloride was dissolved in saline and prepared shortly before use. Control animals received an equivalent volume of saline.

Experimental Design

Initial experiments were conducted to investigate whether the uptake of liposomal α-tocopherol is influenced by the course of paraquat-induced lung injury. Animals in the control group were given an intravenous injection of saline and liposomal α-tocopherol and 24 h later, they were killed and their lungs removed for assay (Fig. 1). Animals in the experimental groups were pretreated with the pulmonary toxicant, paraquat dichloride (20 mg/kg), and 4, 24,

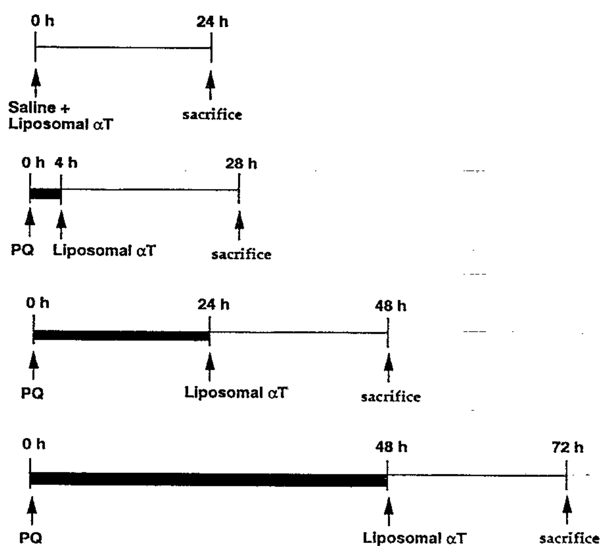

FIGURE 1 Experimental protocol of paraquat dichloride (PQ) injection and liposomal α-tocopherol treatment. Animals were injected i.p. with saline or PQ (20 mg/kg body weight). At the indicated time intervals after PQ injection in each case, animals were intravenously administered with liposomal α-tocopherol (20 mg α-tocopherol/kg body weight). Animals were killed 24 h after liposomal treatment in each case and their lungs removed for analysis.

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or 48 h later were given liposomal α-tocopherol; animals were killed 24 h after the administration of liposomal α-tocopherol in each case (Fig. 1).

To investigate whether the pulmonary uptake of liposomal α-tocopherol was directly related to the extent of residual lung injury, animals were pretreated with different doses of paraquat dichloride (0, 20, 30, or 40 mg/kg body weight) and 48 h later were treated with liposomal α-tocopherol; animals were killed 24 h post-liposomal administration.

The relative extent of paraquat-induced pulmonary endothelial cell and alveolar type II epithelial cell damage was assessed biochemically by measuring the activities of angiotensin converting enzyme (ACE) and alkaline phosphatase (AKP), respectively. The uptake of liposomal α-tocopherol by the normal and injured lung was assessed by measuring $[^3H]$α-tocopherol and $[^{14}C]$DPPC radioactivity in the pulmonary homogenates.

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°C. 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 25% homogenate.

Measurement of Radioactivity

Lung homogenates were digested with 0.5 ml Solvable (DuPont) for 24 h and then bleached with 0.2 ml of 30% H_2O_2. The mixture was subsequently incubated at 25°C for 1 h. After incubation, 10 ml of Formula 989 scintillation fluid (New England Nuclear, DuPont) was added, the samples vortexed, and counted for radioactivity in a Beckman LS 5801 scintillation counter.

Biochemical Analyses

Angiotensin converting enzyme (ACE) activity was determined using the Sigma Diagnostic procedure as described by Suntres et al. (1992). One unit of ACE activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol furylacrylloylphenylalanine per min at 37°C.

Alkaline phosphatase (AKP) activity was determined as described previously. One enzymatic unit was defined as the amount of enzyme that catalyzed the formation of 1 nmol of p-nitrophenol per min (Boudreau and Nadeau, 1987).

Myeloperoxidase (MPO) activity was determined by following the changes in optical density resulted from the decomposition of hydrogen peroxide in the presence of tetramethylbenzidine and was expressed as changes in absorbance measured at 450 nm (Mulligan et al., 1991).

Protein determinations were estimated by the method of Lowry et al. (1951), using crystalline bovine serum albumin as the standard.

Statistical Analysis

Data from control and treated groups were evaluated by one-way analysis of variance (ANOVA). The level of significance was accepted at $p < 0.05$. The correlation coefficient was obtained by the Pearson product-moment correlation method (Gad and Weil, 1994).

RESULTS

Enzyme Markers of Pulmonary Injury

Angiotensin converting enzyme and alkaline phosphatase activities. It has been demonstrated that measurement of angiotensin converting enzyme (ACE) and alkaline phosphatase (AKP) activities could serve as indicators of endothelial cell and alveolar type II epithelial injury, respectively (Lazo et al.,

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1986; Boudreau and Nadeau, 1987). Paraquat dichloride administration produced significant time- and dose-dependent decreases in pulmonary ACE and AKP activities compared to saline-treated animals (Fig. 2 A–B, D–E).

Myeloperoxidase activity. The effect of intraperitoneally administered paraquat dichloride on the activity of myeloperoxidase (MPO), an enzyme marker of inflammatory cell infiltration, in sonicated lung homogenates is shown in Fig. 2 (C, F). Paraquat dichlo-

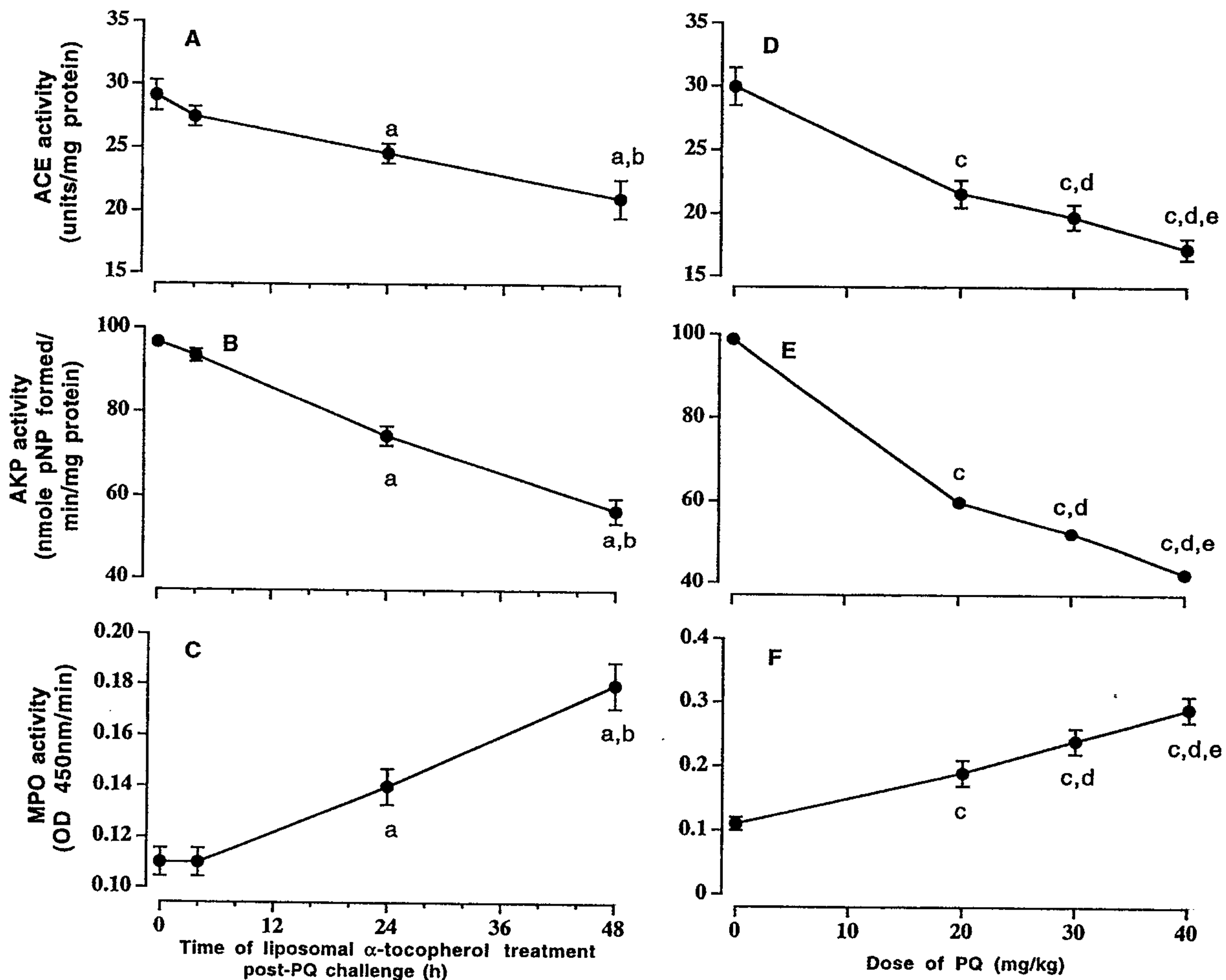

FIGURE 2 Paraquat-induced changes in angiotensin converting enzyme (ACE), alkaline phosphatase (AKP) and myeloperoxidase (MPO) activities in the lung. Animals were treated as described in Fig. 1 and the lungs of treated animals were removed 24 h after liposomal treatment and used for enzymatic assays. Each data point represents the mean ± SEM of 6 animals. [a]denotes a significant difference ($p < 0.05$) compared with the mean value of animals treated with liposomal α-tocopherol 4 h post-paraquat dichloride (PQ) injection. [b]denotes a significant difference ($p < 0.05$) compared with the mean value of animals treated with liposomal α-tocopherol 24 h post-PQ injection. [c]denotes a significant difference ($p < 0.05$) compared with the mean value of control animals injected with saline. [d]denotes a significant difference ($p < 0.05$) compared with the mean value of liposomal α-tocopherol-treated animals pre-injected with a dose of PQ at 20 mg/kg. [e]denotes a significant difference ($p < 0.05$) compared with the mean value of liposomal α-tocopherol-treated animals pre-injected with a dose of PQ at 30 mg/kg.

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ride administration produced a significant time- and dose-dependent increase in pulmonary MPO activity compared to saline-treated animals.

Pulmonary Uptake of Radioactive α-tocopherol Liposomes

The recovery of radiolabelled liposomal α-tocopherol from the lungs of saline-treated or paraquat-treated animals is shown in Table I. The pulmonary recovery of α-tocopherol radioactivity 24 h following administration of liposomal α-tocopherol in saline-treated animals was estimated to be about 2% (108.7 μg α-tocopherol/lung) of the initial dose. The radioactivity in the lungs of rats pretreated with 20 mg/kg paraquat dichloride for 24 and 48 h was significantly increased by 20 and 78%, respectively when compared to saline-treated animals. The recovery of radioactivity of α-tocopherol liposomes in the whole blood of control animals injected with saline and liposomal α-tocopherol was found to be less than 2% of the initial dose (data not shown). The contribution of radioactive counts in the lung from pulmonary blood has been calculated to be less than 0.1% of the initial dose.

To ascertain whether the pulmonary uptake of α-tocopherol liposomes was directly related to the extent of residual lung injury, the effect of different doses of paraquat on the localization of radiolabelled α-tocopherol was examined. As shown in Table II, a dose-dependent increase (by about 2 to 3-fold) of α-tocopherol radioactivity in the lungs of rats was observed following the administration of increasing doses of paraquat dichloride. A linear relationship between pulmonary α-tocopherol uptake and the corresponding residual change in specific lung injury enzyme markers of ACE, AKP and MPO was also evident (Fig. 3).

Comparison of [^{3}H] α-tocopherol and [^{14}C] Liposome Distribution in the Lung

To investigate whether the distribution of α-tocopherol radioactivity in the lung of saline-treated and paraquat-treated animals was directly related to that of ^{14}C-labelled liposomes, the distribution of ^{3}H- and ^{14}C-radioactivity was compared in lung homogenates. As shown in Tables I and II, the uptake of ^{14}C-liposomal lipid was increased in a time- and dose-dependent fashion and was very similar to that observed for α-tocopherol radioactivity, resulting in a practically constant ^{3}H/^{14}C ratio.

DISCUSSION

The pulmonary toxic effects of paraquat are primarily manifested by pulmonary edema, haemorrhage, interstitial inflammation and proliferation of bronchial epithelium, effects that mimic the clinical manifestations of adult respiratory distress syndrome, ARDS (Heath and Smith, 1977; Pasi, 1978). ARDS is known to develop as a complication of various pathological conditions including sepsis, reperfusion of ischemic tissue, trauma and chemically-induced injury (Blennerhassett, 1985; Kehrer and Kacew, 1985; Villar and Slutsky, 1989; McLean and Byrick, 1993). Although

TABLE I Effect of paraquat pretreatment on the pulmonary uptake of radioactive liposomal α-tocopherol

Pretreatment	Time of liposomal α-tocopherol administration after pretreatment (*h*)	24 h post liposomal α-tocopherol administration [^{3}H] α-tocopherol (dpm/lung)	[^{14}C] liposomal lipid (dpm/lung)	Ratio (^{3}H/^{14}C)
Saline	48	718 ± 37	688 ± 43	1.04
Paraquat	4	634 ± 97	693 ± 11	0.91
Paraquat	24	859 ± 34	952 ± 138	0.90
Paraquat	48	1,278 ± 92	1,250 ± 212	1.02

Animals (n = 6 per group) were pretreated with an intraperitoneal injection of saline or paraquat dichloride (20 mg/kg) and at the indicated times later, were given an intravenous injection of [^{14}C]liposome-associated [^{3}H]α-tocopherol. All treated animals were killed 24 h after liposomal α-tocopherol administration and their lungs were removed for analysis.

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TABLE II Effect of different paraquat doses on the pulmonary uptake of radioactive liposomal α-tocopherol

Paraquat dose (*mg/kg*)	Administration of liposomal α-tocopherol 48 h after paraquat	24 h post liposomal α-tocopherol administration		
		[^{3}H] α-tocopherol (*dpm/lung*)	[^{14}C]liposomal lipid (*dpm/lung*)	Ratio ($^3H/^{14}C$)
0	+	718 ± 37	688 ± 43	1.04
20	+	1,278 ± 92	1,250 ± 212	1.02
30	+	1,596 ± 100	1,500 ± 145	1.06
40	+	2,397 ± 271	2,305 ± 190	1.04

Animals (n = 6 per group) were pretreated with an intraperitoneal injection of saline or the indicated dose of paraquat dichloride and 48 h later, were given an intravenous injection of [^{14}C]liposome-associated [^{3}H]α-tocopherol. All treated animals were killed 24 h after liposomal α-tocopherol administration and their lungs were removed for analysis.

the pathophysiology of this process remains poorly understood, there is increasing evidence that reactive oxygen species play a critical role in the development of ARDS and other oxidant-induced lung injuries (Kehrer and Kacew, 1985; McLean and Byrick, 1993; Cross et al., 1994). Paraquat is a very strong oxidant known to cause lethal toxicity in both humans and animals via oxidative stress-mediated mechanisms and it has been shown in this and other studies that

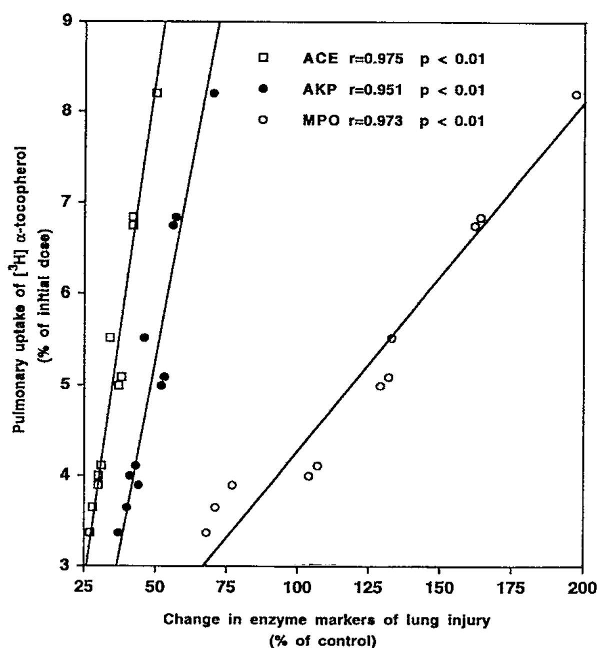

FIGURE 3 Correlation between pulmonary uptake of [^{3}H]α-tocopherol and changes in different enzyme markers of lung injury. Animals were treated as described in Fig. 1. ACE, angiotensin converting enzyme; AKP, alkaline phosphatase; MPO, myeloperoxidase.

paraquat administration results in injury to the pulmonary endothelial and alveolar type II epithelial cells (Heath and Smith, 1977; Pasi, 1978).

Although the prophylactic treatment of animals with liposome-encapsulated superoxide dismutase, catalase, glutathione or α-tocopherol has been shown to protect against oxidant-induced lung injury (Turrens et al., 1984; Padmanabhan et al., 1985; Panus and Freeman, 1988; Ward et al., 1988; Suntres et al., 1992), therapeutic intervention is required after the onset of injury in critical care situations. The effective treatment of oxidant-induced lung injury appears to be dependent on, among other factors, the timing of therapy as well as the attainment of sufficient drug level at the site of action. Injury to the lung as well as other tissues is known to alter the tissue distribution of the therapeutic agent (Bakker-Woudenberg, 1992; Smith and Anderson, 1993; Omri et al., 1994). Indeed, results presented in this study demonstrated that prior injury to the lung by paraquat enhanced the pulmonary uptake of liposomal α-tocopherol.

The calculated ^{3}H/^{14}C ratio of the lung homogenates of all groups examined was practically constant throughout the experimental period. The meaning of this constant ratio is not entirely clear. It may suggest that the residual [^{3}H]α-tocopherol remained associated with [^{14}C]liposomes or liposomal fragments. However, since both α-tocopherol and liposomes have been shown to be capable of interacting and associating with cellular and subcellular membranes (Nakagawa et al., 1980; Suntres et al., 1992), a constant ratio may also simply mean the co-presence of α-tocopherol and residual liposomal lipid each associating with cellular components independent of each

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other. Regardless of the mode of association, the fact remains that the administration of α-tocopherol liposomes is effective in treating lung injuries induced by oxidative stress mechanisms (Suntres and Shek, 1995).

For liposomes to reach tissue cells, they must pass through the capillary lining to the interstitial spaces. In lung tissues, where the capillaries are continuous, the uptake of liposomes is relatively low unless liposomes can pass through endothelial cells or squeeze through spaces between endothelial cells (Poste et al., 1984; Hwang, 1987). The increase in α-tocopherol levels in the lung 72 h after paraquat administration may be explained, at least in part, by a possible increase in capillary permeability because of local tissue injury. Indeed, the decrease in lung ACE activities likely indicates endothelial cell damage and the positive correlation between residual lung injury, in terms of changes in enzyme marker activities, and α-tocopherol uptake is an additional indication of liposomal α-tocopherol leakage from the pulmonary vasculature. Our observations appear to be similar to that of a study where the localization of liposomes, 16 h after intravenous administration, was significantly increased in the lungs of rats infected with *Klebsiella pneumoniae* compared with that of uninfected animals and furthermore, the extent of liposome localization in the lung appeared highly correlated with the intensity of infection (Bakker-Woudenberg et al., 1992). The results of our study, together with those of others, strongly suggest that the enhanced localization of liposomes in injured or infected tissues is the result of extravasation of liposomes at the site of injury or infection, perhaps at the expense of leaky or damaged endothelium.

In order to achieve an effective α-tocopherol level (15–86 μg/lung) for treating lung injury caused by hyperoxia and other oxidative stress-mediated mechanisms, a relatively large dose of α-tocopherol, as large as 100–200 mg/kg, needs to be administered as an aerosol or lipid emulsion (Bucher and Roberts, 1981; Wispe et al., 1986; Hybertson et al., 1993). In contrast, results of this study demonstrated that the intravenous administration of a relatively low dose of liposomal α-tocopherol (20 mg/kg) was sufficient to achieve a relatively high delivery efficiency to the lung (108 μg of α-tocopherol in normal animals and as high as 366 μg in paraquat-treated animals). Thus, the use of a liposomal formulation immensely facilitates the pulmonary delivery of α-tocopherol, an extremely insoluble yet effective oxidant scavenger.

Mortality from ARDS is relatively high and has remained unchanged for the last several decades, despite aggressive supportive therapy. Intervention with pharmacologic agents such as corticosteroids (Bernard et al., 1987) and PGE_1 (Russel, 1987) has been found ineffective, thus necessitating the development of alternative therapeutic interventions. Since reactive oxygen species play a critical role in the development of ARDS, the use of antioxidants as a therapeutic alternative is considered promising. Results of the present study have demonstrated that the intravenous administration of α-tocopherol liposomes can result in a significant elevation of the antioxidant in the injured lung. It is also evident that the extent of liposomal α-tocopherol uptake is directly related to the degree of lung injury. Currently, experiments are underway to assess the efficacy of this formulation in the treatment of lung injury induced by oxidative stress-mediated mechanisms.

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