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Incorporation of α-Tocopherol in Liposomes Promotes the Retention of Liposome-encapsulated Glutathione in the Rat Lung

Z. E. SUNTRES AND P. N. SHEK

Operational Medicine Section, Biosciences Division, Defence and Civil Institute of Environmental Medicine, North York, Ontario M3M 3B9, Canada

Abstract—The present study was undertaken to investigate whether α-tocopherol incorporated in liposomes could improve the retention of entrapped glutathione (GSH) in the lung following intratracheal instillation in rats. Rats were treated with a single dose of [^{3}H]GSH entrapped in liposomes with or without 30 mol% α-tocopherol and killed 0, 24 or 48 h later. The retention of GSH in the lung was assessed by measuring the recovery of either ^{3}H-label or GSH in the lung. Animals instilled with free [^{3}H]GSH were found to retain only 2% of the administered dose at 24 h after treatment and no detectable radioactivity at 48 h. Liposome encapsulation altered the pulmonary retention of GSH with 18 and 10% of radioactivity remaining in the lung at 24 and 48 h post-treatment, respectively. The instillation of GSH encapsulated in α-tocopherol-containing liposomes resulted in the highest level of GSH retention in the lung, namely 37 and 30% of the administered GSH dose at 24 and 48 h, respectively. Results from Sepharose 4B column chromatography revealed that lung homogenates, obtained from rats instilled with GSH entrapped in α-tocopherol-containing liposomes, 24 and 48 h earlier, contained 2 eluted GSH-related components—one associated with the liposomal lipid marker in the void volume and the other as free GSH tripeptide, suggesting a slow sustained release effect mediated by the liposomal formulation. The same liposome preparation containing both α-tocopherol and GSH also conferred better protection against FeADP-induced lipid peroxidation than liposomes containing either α-tocopherol or GSH alone, indicative of a potentially effective antioxidant formulation for treating oxidative lung injury.

The controlled release of liposomal contents is considered one of the most advantageous properties of liposomes as a drug carrier system. The rate and extent of release of an encapsulated drug in the target tissue could influence the onset and duration of the drug's therapeutic effect (Poznansky & Juliano 1984; Patel 1985). Observations from both in-vivo and in-vitro studies, however, have revealed that in most cases after parenteral administration, the entrapped drug may be released from liposomes prematurely upon encountering the biological milieu (Kirby et al 1980a, b; Franson 1981; Hunt 1981; Kaye 1981; Patel 1985). Such instability of liposomal membranes, resulting in the leakage of the entrapped solute, has been attributed to interactions of the liposomal lipid bilayers with various destabilizing serum proteins, degradative enzymes and oxidative agents (Kirby et al 1980a, b; Franson 1981; Hunt 1981; Kaye 1981; Montfoort et al 1987). So far, modulation of factors such as lipid composition, surface charge and vesicle size has, to a large extent, improved the stability of liposomes (Kirby et al 1980b; Hunt 1981; Kaye 1981; Ostro 1987; Defrise-Quertain et al 1984; Gregoriadis 1988). The incorporation of cholesterol, for example, in liposomal membranes has also been shown to enhance liposome stability and decrease solute leakage. Cholesterol is known to influence the permeability of phospholipid vesicles by decreasing their vulnerability to the destructive action of plasma proteins and enzymes (Guo et al 1980; Kirby et al 1980b; Damen et al 1981; Gregoriadis 1988).

Like cholesterol, α-tocopherol is an amphipathic compound consisting of condensed rings bonded separately to hydrophobic aliphatic side chains and a single hydrophilic hydroxyl group. In studies examining the transfer of cholesterol and α-tocopherol between liposomal membranes, it has been demonstrated that α-tocopherol and cholesterol can both influence the fluidity of phospholipid bilayers in a similar manner (Nakagawa et al 1980). Improved stability of liposomes by cholesterol has been extensively investigated both in-vivo and in-vitro (Hunt 1981; Kaye 1981; Ostro 1987; Defrise-Quertain et al 1984; Gregoriadis 1988). However, there has been little or no studies designed to examine the effect of the incorporation of α-tocopherol in liposomes on the retention of liposome-encapsulated water-soluble drugs in-vivo.

The present study was undertaken to investigate whether α-tocopherol incorporated in liposomes could improve the pulmonary retention of an entrapped hydrophilic agent such as glutathione (GSH), in-vivo. The lung was chosen as the organ of interest firstly because the uptake and distribution of liposome-associated α-tocopherol in the lung has been examined (Suntres et al 1993) and secondly because lung tissues are highly susceptible to toxic, oxidative environmental insults (Bend et al 1985; Menzel & Amdur 1986). GSH was used as the entrapped solute because of its therapeutic potential as an antioxidant. Furthermore, GSH is a hydrophilic tripeptide which not only serves well as a liposomal aqueous marker, but also represents a small, water-soluble drug, typically cleared rapidly from the lung (Meister & Anderson 1983; Jurima-Romet et al 1990; Smith et al 1992). Thus, in this report we studied the pulmonary retention of intratracheally administered free GSH and GSH entrapped in liposomes with or without α-tocopherol.

Correspondence: P. N. Shek, Defence and Civil Institute of Environmental Medicine, 1133 Sheppard Avenue West, North York, Ontario M3M 3B9, Canada.

Materials and Methods

Chemicals

Glutathione and α-tocopherol were purchased from Sigma Chemical Co. (St Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL). [^{3}H]GSH and [^{14}C]cholesterol were purchased from New England Nuclear, Dupont, Canada (Mississauga, Ontario). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO) or BDH (Toronto, Ontario). Sepharose 4B column was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Animals

Male Sprague-Dawley rats, 220–250 g, were purchased from Charles River Canada Inc. (St Constant, Quebec). 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 research were cared for in accordance with the principles contained in the Guide to the Care and Use of Experimental Animals as prepared by the Canadian Council on Animal Care.

Experimental design

In-vivo study. Animals were treated intratracheally with a single dose of [^{3}H]GSH entrapped in liposomes with or without α-tocopherol and killed 0, 24, or 48 h later. The retention of GSH in the lungs of treated animals was determined by measuring the recovery of ^{3}H and GSH concentration in the lung homogenate.

In-vitro study. Lungs were obtained from animals immediately after treatment with liposome-entrapped GSH, α-tocopherol-containing liposomes with entrapped GSH and α-tocopherol-containing liposomes without GSH. Standard incubations were carried out in a 25 mL Erlenmeyer flask with a final volume of 5 mL of incubation mixture containing 1 mL of 20% lung homogenate, 10 mM NADPH, 50 mM $MgCl_2$ and 30 mM Tris-HCl buffer, pH 7·4. The concentrations of GSH and α-tocopherol in the lung homogenates of untreated animals were determined to be 0·24 and 0·03 μmol mL^{-1}, respectively. The concentration of GSH in the lung homogenates of animals treated with liposome-entrapped GSH and α-tocopherol-liposome-entrapped GSH was estimated to be 1·21 μmol mL^{-1}. The concentration of α-tocopherol in lung homogenates of animals treated with α-tocopherol-liposomes with entrapped GSH and α-tocopherol-liposomes without GSH was estimated to be 0·46 μmol mL^{-1}. The incubation was carried out at 37°C with air as the gas phase in a metabolic shaker oscillating at 100 cycles min^{-1}. After a 3-min pre-incubation period, the reaction was initiated by the addition of FeADP to give a final concentration of 200 μM Fe^{3+}/0·8 mM ADP in the incubation mixture. Aliquots of the mixture were removed at different times and the reactions were terminated by the addition of ice-cold 10% trichloroacetic acid. The samples were centrifuged at 600 *g* for 10 min and the supernatant fractions were used for the measurement of lipid peroxidation by measuring the formation of thiobarbituric acid reactants.

Preparation of liposomal suspensions

The procedure for the entrapment of GSH in DPPC liposomes was performed as previously described (Jurima-Romet & Shek 1991) and liposomes containing α-tocopherol were prepared from a mixture of DPPC and α-tocopherol in a 7:3 molar ratio. Briefly, the lipids were dissolved in chloroform-methanol (2:1, v/v) and the lipid mixture was dried in a waterbath at 40°C under a stream of helium to a thin film, coating the interior surface of a glass vessel. Any traces of solvent were removed by placing the vessel under vacuum for at least 1 h. The dried lipid was hydrated with 1 mL of 5 mM potassium phosphate buffer, pH 6·5, containing 0·4 M GSH, and then vortexed to form multilamellar vesicles. The multilamellar vesicles were extruded (10 times) with an extruder (Lipex Biomolecules, Vancouver, BC), fitted with two stacked polycarbonate filters of 4000-nm pore size, using a helium pressure of 100–200 psi. Free GSH was removed by washing the liposomes twice in 5 mM potassium phosphate buffer, pH 6·5, and pelleting at 110000 *g* for 1 h at 5°C in a Beckman L8-70 ultracentrifuge. Supernatant and pellet fractions were counted for ^{3}H and ^{14}C to determine the GSH entrapment efficiency. Liposomal vesicle size was determined with the use of a Coulter N4SD particle-size analyser and was found to have a mean diameter of 290 ± 35 nm.

Treatment of animals

Intra-tracheal instillation of free GSH or GSH-entrapped in liposomes in rats was performed by adopting the endotracheal intubation procedure described by Brain et al (1976). Each animal received 150 μL of instillate containing 9 μmol of either free or liposome-encapsulated GSH between 0800–0900 h.

Preparation of tissues

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 weighed and finely minced. Approximately 1 g lung sample was homogenized with a Brinkman Polytron in a sufficient volume of ice-cold 50 mM potassium phosphate buffer, pH 7·4, to produce a 20% homogenate. The homogenate was centrifuged at 9000 *g* for 10 min in a refrigerated Sorvall RC-5B centrifuge. The post-mitochondrial supernatant was decanted and re-centrifuged at 105000 *g* for 60 min in a refrigerated Beckman L8-70 ultracentrifuge to obtain the microsomal and cytosolic fractions.

Determination of lipid peroxidation

Lung homogenates from treated animals were assayed for the presence of thiobarbituric acid reactants (Suntres & Lui 1990).

Determination of pulmonary GSH content

Non-protein sulphydryl concentrations in lung homogenates were determined as described by Suntres & Lui (1991). Briefly, the tissue was homogenized in 20% (w/v) trichloroacetic acid and centrifuged at 10000 rev min^{-1} for 20 min in a refrigerated Sorvall RC-5B centrifuge. An aliquot of the deproteinized supernatant fraction was added to 2 mL 0·3 M Na_2HPO_4 solution followed by the addition of 0·5 mL

Table 1. Recovery of ^{3}H and ^{14}C from lung homogenates of rats following intratracheal instillation of different GSH formulations.

GSH formulation used for treatment	% Radioactivity			
	Total ^{3}H recovered		Total ^{14}C recovered	
	24 h	48 h	24 h	48 h
GSH solution	2±1	0	—	—
GSH in liposomes	18±4	10±2	83±4	75±5
GSH in α-tocopherol-liposomes	37±5	30±4	85±6	78±4

Different groups of rats were administered intratracheally with the indicated GSH formulations according to the procedures described in Materials and Methods. The recovery of ^{3}H and ^{14}C labels from lungs of treated rats was each determined at 24 and 48 h, respectively, after treatment. Each value represents the mean percentage of recovered dose ± s.e.m. of four animals. In terms of ^{3}H recovery, there is a statistically significant ($P < 0.05$) difference among the corresponding values of the three groups of treated animals, at the 24- and 48-h time-points, respectively.

Table 2. Pulmonary GSH contents in rats following intratracheal administration of different GSH formulations.

GSH formulation used for treatment	GSH content (μmol (lung)$^{-1}$)		
	0 h	24 h	48 h
GSH solution	12·4±1·0	0·2±0·4 (2%)*	0·2±0·1 (2%)*
GSH in liposomes	12·0±0·6	1·9±0·4 (16%)*	1·2±0·3 (10%)*
GSH in α-tocopherol-liposomes	12·0±0·5	4·1±0·4 (34%)*	3·1±0·4 (26%)*

Different groups of rats (n = 4 per group) were administered intratracheally with the indicated GSH formulations according to the procedures described in Materials and Methods. Animals were killed and their lungs removed at 0, 24 and 48 h after treatment for the determination of GSH content. The results are expressed as net GSH content (total measurable GSH in treated lungs − mean GSH content in untreated lungs) ± s.e.m. remaining in the lung after intratracheal instillation. Each bracketed number shows the GSH content as a percentage of the initial dose (time zero). * $P < 0.05$ in GSH content compared to the corresponding time-zero value.

0·04% 5,5-dithiobis-[2-nitrobenzoic acid] dissolved in 10% sodium citrate. The absorbance at 412 nm was measured immediately after mixing.

GSH was also assayed after 1 mL deproteinized supernatant fraction was treated with sodium borohydride to reduce GSSG to GSH at 45°C for 1 h.

Chromatographic analysis

A Sepharose 4B column (1·5 cm × 26 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6·8, was used for the fractionation of lung homogenates to trace the appearance of the ^{14}C (lipid) and ^{3}H (glutathione) markers. A volume of 0·5 mL of the lung homogenate (20%) sample was passed through the column and fractions of 1 mL were collected and analysed for radioactivity.

Statistical analysis

All data obtained from control and experimental groups were analysed by one-way analysis of variance. If the F values were significant, the unpaired two-tailed Student's *t*-test was used to compare the mean values of appropriate experimental groups (Gad & Weil 1982). The level of significance was accepted at $P < 0.05$.

Results

Pulmonary recovery of ^{3}H and ^{14}C labels

The results are shown in Tables 1 and 2. The relative concordance of the recovery data between Tables 1 and 2 strongly suggested that the ^{3}H radioactivity recovered represented, to a large extent, the active glutathione tripeptide molecule. A similar retention of ^{14}C-label (about 75–85% of initial dose) was observed in the lungs of rats instilled with GSH encapsulated in liposomes with or without α-tocopherol at 24 and 48 h post-treatment (Table 1). Determination of internal-space/bilayer marker ratio has been suggested to provide a good indication of liposomal integrity (Mayhew & Papahadjopoulos 1983). In this study, the internal-space marker/bilayer marker ($^{3}H/^{14}C$) ratio (0·44 and 0·40) observed in the lungs of animals treated with GSH encapsulated in liposomes containing 30 mol% α-tocopherol was higher than the ratio (0·21 and 0·13) of animals treated with GSH encapsulated in α-tocopherol-free liposomes, at 24 and 48 h, respectively. These data suggested that the incorporation of α-tocopherol in the liposomal membrane enhanced the retention of the entrapped GSH in the lung.

Chromatographic analysis of lung homogenates

The elution of homogenate samples isolated from rat lungs 24 or 48 h after the instillation of GSH encapsulated in α-tocopherol-free liposomes revealed that [^{3}H]GSH was co-eluted with the ^{14}C-label as a single peak in the void volume of the column (Fig. 1A, B); these data suggested that the GSH present in the lung was primarily associated with liposomes. In contrast to the unimodal distribution of ^{3}H,

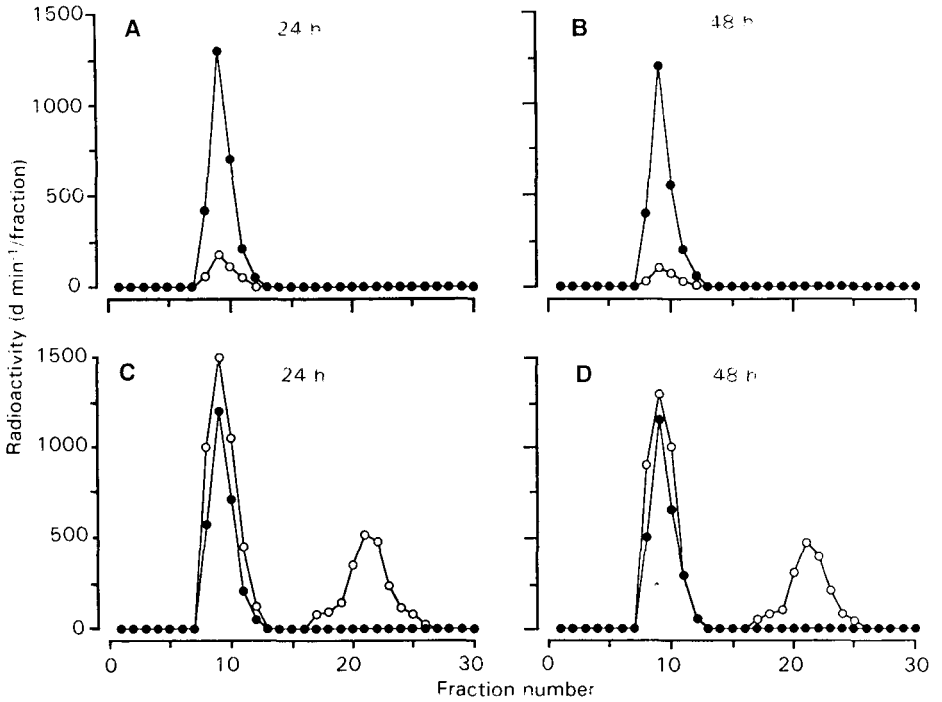

FIG. 1. Chromatographic analysis of lung homogenates prepared from lungs of rats 24 or 48 h after intratracheal instillation of GSH encapsulated in DPPC liposomes (A, B) or GSH encapsulated in α-tocopherol-containing DPPC liposomes (C, D). Homogenates, prepared from lung tissues pooled from four animals, were chromatographed on a Sepharose 4B column (1·5 × 26 cm), equilibrated with 10 mM potassium phosphate buffer, pH 6·8, at room temperature (21°C). Fractions (1 mL) were collected at an elution rate of 1 mL min^{-1} and each fraction was measured for ^{3}H (○——○, GSH-label) and ^{14}C (●——●, liposomal lipid-label) radioactivity.

similar chromatographic analysis of homogenates isolated from the lungs of rats instilled with [^{3}H]GSH encapsulated in liposomes containing α-tocopherol showed a bimodal distribution (Fig. 1C, D). In addition to the co-elution of [^{3}H]GSH with ^{14}C-liposomal label in the void volume, a second ^{3}H peak was also recovered in fractions 17–25, corresponding to an identical elution profile of free GSH (data not shown). A comparison of ^{3}H in the excluded peaks (Fig. 1A, C) also revealed a substantially higher retention of GSH in α-tocopherol-containing liposomes than α-tocopherol-free liposomes. These data appeared to suggest that the incorporation of α-tocopherol in phospholipid membranes of liposomes enabled a slower sustained release of the entrapped solute.

Resistance of lung homogenates to lipid peroxidation in-vitro

To examine the resistance of lung homogenates to oxidative challenge, the extent of FeADP-induced lipid peroxidation was measured. For the preparation of homogenates, lungs were obtained from control rats receiving no treatment and from rats treated with liposomes containing α-tocopherol, liposomes containing GSH, or liposomes containing both GSH and α-tocopherol. As shown in Fig. 2, the lung homogenates from control animals with no treatment were least resistant to lipid peroxidation. Homogenates obtained from rats instilled with antioxidant-containing liposomes were all significantly more resistant to peroxidation than those from control animals. Liposomes containing α-tocopherol were better than liposomes containing GSH in terms of resistance to peroxidation. The most effective formulation

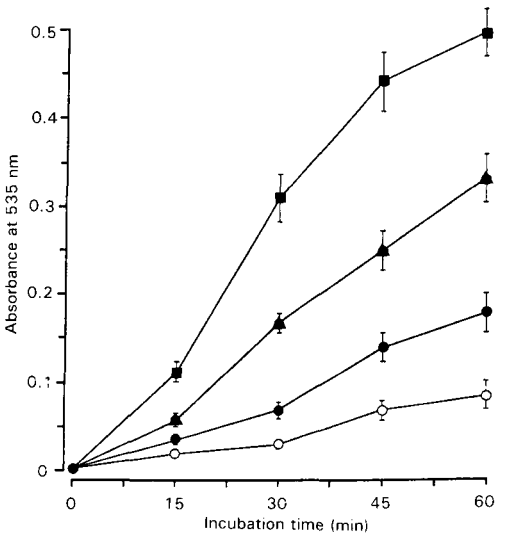

FIG. 2. Fe-ADP-induced lipid peroxidation in lung homogenates prepared and obtained from rats which were either untreated (■) or instilled with liposome-entrapped GSH (▲); α-tocopherol-containing liposomes without GSH (●); and α-tocopherol-containing liposomes with entrapped GSH (○). Lungs were obtained from the animals immediately after treatment and incubations were carried out as described in Materials and Methods. Each point represents the mean ± s.e.m. of three experiments. At each of the indicated incubation periods (15, 30, 45 and 60 min), differences among the corresponding values obtained from the incubations of lung homogenates of the four groups of animals were determined to be all statistically significant ($P < 0.05$).

in protecting the lung from FeADP-induced lipid peroxidation was the liposome preparation containing both GSH and α-tocopherol. The data of Fig. 2 represent lipid peroxidation results of lung tissues obtained from rats immediately after treatment; similar differences in the magnitude of resistance to FeADP-induced lipid peroxidation were also observed among lung homogenates obtained from animals killed 24 or 48 h after instillation of the different formulations (data not shown).

Discussion

The present study has demonstrated that the incorporation of α-tocopherol in liposomal membranes can enhance the retention of entrapped GSH in the lungs of rats, intratracheally instilled with the liposomal preparation. These observations are consistent with the observations of an in-vitro study which demonstrated that the incorporation of α-tocopherol in liposomal membranes retarded the diffusion of a small mol. wt solute, carboxyfluorescein (Halks-Miller et al 1985).

Intratracheally administered free GSH is known to be cleared very rapidly from the lung and, in this study, only a background GSH level remained in the lung by 24 h post-treatment. In an earlier study, we have shown that almost all of the administered free GSH was removed from the lung within 6 h of treatment (Jurima-Romet et al 1990). This rapid clearance is quite typical of small, water-soluble compounds administered to the lung. In this study, we have also demonstrated that the liposomal formulation can be improved by the incorporation of α-tocopherol, allowing for an enhancement of pulmonary GSH retention to 34 and 26% by 24 and 48 h, respectively. The higher retention of entrapped solute in the lung suggested that α-tocopherol may confer a stabilizing effect on the liposomal membrane whereby leakage becomes reduced.

Since α-tocopherol and cholesterol share very similar physicochemical and stabilizing properties (Nakagawa et al 1980), a direct comparison of the effect of the two compounds on the retention of liposome-entrapped solute in the lung would be of much interest. In this regard, the intratracheal administration of GSH entrapped in liposomes containing 30 mol% α-tocopherol resulted in the retention of 37 and 30% of the initial GSH label in the lung at 24 and 48 h, respectively (Table 1); such retention was about twice as much as that (18 and 15% at the corresponding 24 and 48 h time-points) achieved by the administration of GSH entrapped in liposomes containing 30 mol% cholesterol (Jurima-Romet et al 1990). These observations in-vivo are consistent with an in-vitro study which demonstrated that α-tocopherol, even at a lower molar ratio, was more effective than cholesterol in protecting liposomes from protein-induced destabilization (Halks-Miller et al 1985). Thus, the incorporation of α-tocopherol in liposomal bilayers appears useful in preventing the premature release of an entrapped solute, a desirable advantage for the delivery of drugs with relatively short biological half-lives.

The therapeutic efficacy of a pharmacological agent depends on the concentration of the free agent available at the site of action. It has been observed that free GSH, instilled intratracheally into the lungs of rats, is ineffective in the treatment of oxidant-induced lung damage (Meister & Anderson 1983; Smith et al 1992) because of its rapid clearance from the lung (Jurima-Romet et al 1990; Smith et al 1992). Results of this and other studies have shown that encapsulation of GSH in liposomes prolongs its retention in the tissue (Jurima-Romet et al 1990; Smith et al 1992) and confers protection against oxygen-induced lung damage (Smith et al 1992). Although the exact mechanism whereby liposome-encapsulated GSH protects against hyperoxia in rats is not known, it has been suggested that the prolonged retention of liposomes in the lung perhaps enables the slow release of the GSH content to key lung cells (Smith et al 1992). This suggestion is supported by our chromatographic results which substantiated the availability of free GSH, presumably released from the liposomal pool, in lung homogenates of rats instilled with GSH entrapped in α-tocopherol-containing liposomes. The liposome-mediated sustained-release effect is indicated by the detection of free GSH in the lung as late as 24 and 48 h post-intratracheal instillation.

Liposomes containing both α-tocopherol and GSH were more effective in protecting against FeADP-induced membrane lipid peroxidation than those containing either α-tocopherol or GSH alone. It has been shown that α-tocopherol can exert an antioxidant effect by scavenging free radicals and stabilizing biological membranes (Tapel 1972; McCay & King 1980) while GSH, in addition to its ability to act as a free-radical scavenger and as a substrate in the glutathione peroxidase/reductase detoxication pathway (Comporti 1987; Horton & Fairhurst 1987), can also regenerate α-tocopherol from its oxidation products (McCay et al 1989; Scholz et al 1989). The incorporation of α-tocopherol in liposomes may therefore serve as a valuable antioxidant drug delivery system.

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