(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 January 2001 (18.01.2001)

PCT

(10) International Publication Number
WO 01/03669 A2

(51) International Patent Classification[7]: A61K 9/127, 31/198, 31/355

(21) International Application Number: PCT/CA00/00806

(22) International Filing Date: 7 July 2000 (07.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/142,545 7 July 1999 (07.07.1999) US

(71) Applicant *(for all designated States except US)*: **HER MAJESTY THE QUEEN as represented by THE MINISTER OF NATIONAL DEFENCE OF HER MAJESTY'S CANADIAN GOVERNMENT** [CA/CA]; Directorate of Intellectual Property, National Defence Headquarters, 101 Colonel by Drive, Ottawa, Ontario K1A 0K2 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHEK, Pang, N.** [CA/CA]; 180 Harlandale Avenue, Toronto, Ontario M2N 1P4 (CA). **SUNTRES, Zacharias, E.** [CA/CA]; 62 Hartsdale Drive, Toronto, Ontario M9R 2S7 (CA). **FAN, Jie** [CA/CA]; 222-22 Elkhorn Drive, Toronto, Ontario M2K 1J4 (CA). **ROTSTEIN, Ori, D.** [CA/CA]; 14 Bennington Heights, Toronto, Ontario M4G 1A6 (CA).

(74) Agent: **SHARPE, Paul, S.**; Marks & Clerk, P.O. Box 957, Station B, Ottawa, Ontario K1P 5S7 (CA).

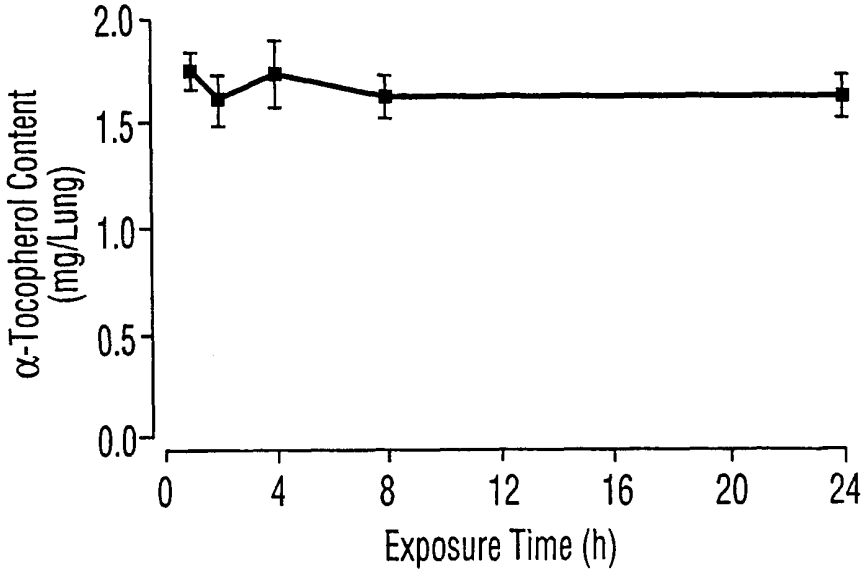
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DELIVERY OF LIPOSOMAL-ENCAPSULATED ANTIOXIDANTS AND APPLICATIONS THEREOF



(57) Abstract: A liposomal composition comprises a hydrophilic sulfhydryl agent and a lipophilic antioxidant, e.g., N-acetyl cysteine and/or α-tocopherol, and a method of delivery by contacting a pulmonary tissue of a mammal with the liposomal composition to achieve a prolonged antioxidant effect.

WO 01/03669 A2

DELIVERY OF LIPOSOMAL-ENCAPSULATED ANTIOXIDANTS AND APPLICATIONS THEREOF

FIELD OF THE INVENTION

The present invention is related to the field of liposome-encapsulation of hydrophilic and hydrophobic agents. More specifically, the present invention relates to the use of liposome-encapsulated antioxidants in the amelioration of pulmonary and hepatic damage in multiple organ dysfunction syndrome (MODS), as well as respiratory distress syndromes of various types and etiologies.

BACKGROUND OF THE INVENTION

Massive hemorrhage is one of the leading causes of mortality in cases of penetrating trauma. For example, the hypoxia within tissues which is associated with hemorrhage and hemorrhagic shock can cause serious damage to endothelial cells. Reperfusion of such ischemic tissue can actually exacerbate the condition, by further promoting the generation of reactive oxygen species, initially by the enzymatic action of xanthine oxidase on xanthine, and later by the recruitment of neutrophils. *See, e.g.,* Granger, 1988. *Am. J. Physiol.* 255: H1269-H1275; Weiss, 1989. *New Engl. J. Med.* 320: 365-376. Although the body is equipped with an anti-oxidant system which possesses the ability to counteract a limited oxidative insult, if the oxidative burden is overwhelming (*e.g.,* in the case of extensive reperfusion injury) the endogenous antioxidant defense simply cannot cope with the extraordinary damaging oxidative load. This condition commonly referred to as oxidant stress.

In addition, in cases of penetrating trauma (*e.g.,* such as those encountered in accident victims or combat casualties), microbial- and toxin-based contamination is frequently encountered. This contamination causes a "double-insult" type of clinical scenario, namely concomitant massive hemorrhage and microbial toxic contamination. Despite intervention with blood replacement and aggressive antibiotic administration, the patient may still develop an uncontrolled, systemic inflammatory response, the severity of which appears to correlate with the development of multiple organ dysfunction syndrome (MODS). *See,* Marshall and Sweeney, 1990. *Arch. Surg.* 125: 17-23. The lung is among the earliest and most frequently affected organ in critically ill patients developing MODS. The severity of dysfunction ranges from mild hypoxemia to

a profound respiratory failure designated - Adult Respiratory Distress Syndrome (ARDS).

ARDS is clinically-characterized by hypoxemia, reduced lung compliance, and diffuse alveolar infiltrates. The syndrome usually manifests itself within 2-3 days of the initial underlying disease process, where pro-inflammatory mediators and cells are known to be involved. For example, infiltrating alveolar neutrophils may release reactive oxygen species (*i.e.*, free radicals) and various proteolytic enzymes, causing damage to the endothelium and the epithelium. The invading neutrophils may also either directly release, or initiate the release of, a large number of pro-inflammatory molecules, thus promoting further cellular sequestration and injury. *See.* Murray, *et al.*, 1988. *Am. Rev. Respir. Dis.* 138: 720-723.

ARDS is a frequent complication of sepsis and trauma. The mortality rate associated with this acute lung injury is in the range of 50-70%, and the overall annual incidence of ARDS within the United States has been reported to be no less than 150,000. *See,* Kirkpatrick, *et al.*, 1996. *Shock* 6: S17-S22, 1996. The terminal mediators of the pathophysiological changes associated with ARDS are believed to be reactive oxygen species, which are either generated by *de novo* synthesis at injured tissue sites or released in large concentrations by infiltrating neutrophils. In humans, the extent of neutrophil influx and the presence of neutrophil products in the alveolar lavage fluid have been correlated with the severity of the lung injury. *See,* Hanson, *et al.*, 1984. *Fed. Proc.* 43: 2799.

These aforementioned observations have led to the development of several therapeutic strategies which are designed to both reduce the influx of neutrophils and to counteract the damaging effects of reactive oxygen species. N-acetyl cysteine (NAC), a known free-radical scavenger and anti-oxidant (*see, e.g.*, Gressier, *et al.*, 1994. *Methods Find. Exp. Clin. Pharmacol.* 16: 9-13) has been shown to confer protective effects in endotoxemia (*see, e.g.*, Zhang, *et al.*, 1994. *Am. J. Physiol.* 266: H1746-H1754); to reduce neutrophil influx and lung leaks (*see, e.g.*, Leff, *et al.*, 1993. *Am. J. Physiol.* 265: L501-L506; and attenuate LPS-induced acute lung injury (*see, e.g.*, Davreux, *et al.*, 1997. *Shock* 6: 432-438) in animals. In each of the above-referenced scenarios, however, a very high dose of N-acetyl cysteine (*i.e.*, 150 mg/kg intravenously, or 1 g/kg intraperitoneally) was required in order to achieve a demonstrable biological effect. Moreover, the potential therapeutic application of N-acetylceyteine in humans has been

examined in at least two clinical trials, with markedly different experimental results. The results of one human trial showed *no* beneficial treatment effect (*see,* Jepsen, *et al.*, 1992. *Crit. Care. Med.* 20: 918-923), whereas the other trial demonstrated that the administration of NAC significantly reduced the extent of pulmonary dysfunction (*see,* Suter, *et al.*, 1994. *Chest* 105: 190-194).

Another therapeutic strategy has been to use a very potent lipophilic antioxidant (*e.g.*, α-tocopherol) to quench reactive oxygen species, which mediate the injury at the site of inflammation. It has been demonstrated that α-tocopherol protects against oxidant-induced tissue injury by inhibiting membrane lipid peroxidation and lipid peroxide formation; via scavenging singlet oxygen and other reactive oxygen species and by exerting a stabilizing effect on membranes. *See, e.g.,* Burton and Ingold, 1989. *Ann. N.Y. Acad. Sci.* 570: 7-22. However, α-tocopherol, in its free-form, is too viscous for parenteral administration and emulsifiers which are utilized to solublilize this anti-oxidant are generally found to be toxic to tissues.

Thus, there remains an, as yet, unfulfilled need, for the development of a therapeutic for the treatment of Adult Respiratory Distress Syndrome (ARDS) by mitigating the influx of neutrophils and the damaging effects of pro-inflammatory mediators (*e.g.*, reactive oxygen species and various proteolytic enzymes).

SUMMARY OF THE INVENTION

The invention features a therapeutic liposomal formulation, which is useful to ameliorate oxidative tissue damage. Such pathological conditions may be induced by hemorrhagic shock and endotoxin insult. The liposomal formulation of the invention is suitable for circulatory (*e.g.*, intravenous) or intratracheal administration, and has been quantitatively demonstrated to ameliorate lung and liver injuries caused by neutrophil infiltration and reactive oxygen species. The compositions and methods are also useful for reducing the extent of complications associated with the clinical condition of ARDS and liver injuries.

Accordingly, the invention provides a liposomal composition containing a hydrophilic sulfhydryl agent and a lipophilic antioxidant. Preferably the composition contains at least 1%, more preferably at least 10%, more preferably at least 20%, more preferably at least 25% by weight of the hydrophilic agent. For example, the composition contains 28% by weight of a hydrophilic sulfhydryl agent. The amount of

hydrophilic sulfhydryl agent in the composition does not exceed 50% by weight.

The composition is characterized as having free radical scavenging activity and antioxidant activity. The term "free-radical", as utilized herein, is defined as a reactive chemical intermediate form of an oxygen molecule. For example, a free-radical is $[O_2]^-$ which, due to its high reactivity, can irreversibly damage organic compounds within cells. The term "antioxidant", as utilized herein, is defined as a chemical compound which possesses the ability to mitigate oxidation. For example, an antioxidant reduces the level of oxidation of biological tissues by highly reactive free radicals by neutralizing free radicals. Antioxidants fall into at least two classes: (*i*) endogenously-produced enzyme anti-oxidants (*e.g.*, superoxide dimutase (SOD); glutathione peroxidase) which can catalytically alter or destroy free radicals; and (*ii*) exogenously-ingested nutrients or agents (*e.g.*, α-tocopherol (vitamin E); vitamin C, and β-carotene (vitamin A)) which function to bind and sequester free radicals. Each of the antioxidants listed above may be incorporated into the therapeutic compositions described herein. The antioxidants to be administered are substantially pure, i.e., purified from substances with which they naturally occur.

The compositions contain liposomes, e.g., in the form of unilamellar and oligolamellar liposome vesicles. The range of size of liposomes in the composition is within 25% of the mean size of the liposomes. The liposomes of the composition are relatively uniform in size. For example, the range of size of liposomes in the composition is preferably within 20%, more preferably within 15%, more preferably within 10%, and most preferably within 5% of the mean size of the liposomes. For example, at least 85% (more preferably 90%, more preferably 95%, and most preferably 99-100%) of the liposomes in the composition are with a defined size range, e.g., between 100-400 nm in size. To produce uniformly-sized liposomes, the vesicles are produced by extrusion rather than sonication. Liposomes are extruded to be approximately 150 nm in size. In another example, the liposomes are extruded to be approximately 450 nm in size. Unlike other methods of liposome manufacture (*e.g.*, sonication which method yields a heterogeneous population of liposomes which vary widely in size), extrusion yields a population of liposomes that are relatively uniform in size. Uniformity of size allows more reproducible pharmacokinetics than other methods in the art. The hydrophilic sulfhydryl agent is encapsulated in an aqueous interior of a liposomal vesicle and the lipophilic antioxidant is incorporated an outer membrane of

the liposomal vesicle.

The hydrophilic sulfhydryl agent preferably is an antioxidant such as N-acetyl cysteine, and the lipophilic antioxidant is preferably α-tocopherol. The composition contains at least 1%, more preferably at least 5%, more preferably at least 7%, and most preferably at least 9% by weight of the lipophilic antioxidant. The composition may also contain a phospholipid such as a phosphatidylcholine, a dipalmitoylphosphatidylcholine, a lysophosphatidylcholine, a phosphatidylserine, a phosphatidyl-ethanolamine, a phosphatidylglycerol, or a phosphatidylinositol. Cholesterol may also be present in the composition. However, the composition preferably does not contain a metal such as Zn, Se, Cr, Cu, or Mn. For example, the composition is substantially free of such metals which may contaminate a liposomal preparation as a consequence of the sonication process. Since the liposomal compositions described herein are prepared by extrusion rather than sonication, contamination by trace amounts of metals is avoided.

The liposomal composition contains a hydrophilic sulfhydryl agent, a phospholipid, and cholesterol, and the approximate molar ratio of dipalmitoylphosphatyidylcholine:cholesterol is 7:3, 6:4, or 9:1. For example, the phospholipid is dipalmitoylphosphatyidylcholine and the approximate molar ratio of dipalmitoylphosphatyidylcholine:cholesterol is 7:3. In another example, the the approximate molar ratio of phospholipid:cholesterol:hydrophilic sulfhydryl agent is 7:3:15. The liposomal composition is formulated to contain hydrophilic sulfhydryl agent, a phospholipid, cholesterol, and a lipophilic antioxidant, with an approximate molar ratio of phospholipid:lipophilic antioxidant:cholesterol:hydrophilic sulfhydryl agent of 7:2:1:15.

The approximate molar ratio of phospholipid to cholesterol is altered to achieve a desired pharmacokinetic effect. The rate of antioxidant release from the composition is indirectly proportionate to the concentration of cholesterol in the composition, i.e., a higher percentage of cholesterol yields a composition with a slower pharmacokinetic release profile compared to a composition with a lower percentage of cholesterol. Increasing the amount of cholesterol in the composition results in production of liposomes with a more rigid membrane. A more rigid membrane indicates a relatively more stable liposome. A composition formulated with an approximate molar ratio of dipalmitoylphosphatyidylcholine:cholesterol of 7:3 is systemically released over a

longer period of time compared to formulations with a lower relative amount of cholesterol. The compositions contain at least 10% cholesterol. To tailor the kinetics of drug release, the composition is formulated to contain at least 20%, 25%, 30%, 35% or 40% cholesterol. Preferably, the percentage of cholesterol in the composition does not exceed 45%.

The compositions are therapeutically active and have been demonstrated to produce clinical benefits in subjects suffering from oxidative tissue damage. Accordingly, the invention provides a method of delivering an antioxidant to a vertebrate (*e.g.*, a mammal) by contacting a pulmonary tissue of the mammal with a liposomal composition containing a hydrophilic sulfhydryl agent and a lipophilic antioxidant as described above. Preferably, the mammal is a human. The subjects to be treated include those which have been identified as suffering from or at risk of developing a pulmonary injury, a hepatic injury, hemorrhagic shock, endotoxic insult, reperfusion injury, or adult respiratory distress syndrome. Methods of diagnosing such ailments are known in the art. The compositions are administered orally or parenterally, *e.g.*, by an intratracheal, intravenous, intraarterial, intraperitoneal, or intratissue route. The invention also includes a method of treatment for insults of oxidative stress and neturophil infiltration induced by hemorrhagic shock and bacterial lipopolysaccharide challenge. The methods result in a demonstrable reduction of lung and liver injuries.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF THE DRAWINGS

FIGS. 1A-B are line graphs showing the recovery of α-tocopherol and N-acetyl cysteine (NAC) from lung homogenates following the intratracheal instillation of liposomal α-tocopherol (FIG. 1A) or free NAC, liposomal NAC (L-NAC) or liposomal α-tocopherol and NAC (L-αT-NAC) (FIG. 1B). The liposomal preparations were formulated as described in "Preparation of liposome-associated antioxidants", and lungs of treated animals were removed at various time periods after intratracheal instillation as indicated in the figure. Each point represents the mean percentage of recovered dose ± SEM of 4 animals.

FIGS. 2A-B are bar graphs showing the effects of free N-acetyl cysteine (NAC),

liposomal NAC (L-NAC), liposomal α-tocopherol (L-αT), or liposomal α-tocopherol and NAC (L-αT-NAC), administered intratracheally to the lungs of shocked animals. FIG. 2A shows changes on the LPS-induced changes in lipid peroxidation, an indicator of oxidative stress, and FIG. 2B shows changes in non-protein thiol concentration, a group of protective agents against oxidant-induced injury. Animals were maintained in the hemorrhagic shock state for 60 minuets, followed by reperfusion with shed blood over a 2 hour period. Thirty minutes after reperfusion, animals were intratracheally instilled with saline or different antioxidant preparations. Following a period of 18 hours after the initiation of shock, animals were challenged intratreacheally with LPS (300 μg/kg body weight) and killed 4 hours later. Each data point represents the mean ± SEM of 6 animals. The symbol "*" represents significantly different ($p<0.05$) from the corresponding value obtained from shocked animals treated with saline and challenged with LPS.

FIGS. 3A-B are bar graphs showing the effects of free N-acetyl cysteine (NAC), liposomal NAC (L-NAC), liposomal α-tocopherol (L-αT), or liposomal α-tocopherol and NAC (L-αT-NAC), administered intratracheally to the lungs of shocked animals. FIG. 3A shows changes on the LPS-induced changes in pulmonary myeloperoxidase concentration, and FIG. 3B shows the number of polymorphonuclear leukocytes (PMN) in the bronchoalveolar lavage (BAL) fluid. Animals were maintained in the hemorrhagic shock state for 60 minuets, followed by reperfusion with shed blood over a 2 hour period. Thirty minutes after reperfusion, animals were intratracheally instilled with saline or different antioxidant preparations. Following a period of 18 hours after the initiation of shock, animals were challenged intratracheally with LPS (300 g/kg body weight) and killed 4 hours later. Each data point represents the mean ± SEM of 6 animals. The symbol "*" represents significantly different ($p<0.05$) from the corresponding value obtained from shocked animals treated with saline and challenged with LPS.

FIG. 4 is a bar graph showing the effects of free N-acetyl cysteine (NAC), liposomal NAC (L-NAC), liposomal α-tocopherol (L-αT), or liposomal α-tocopherol and NAC (L-αT-NAC), administered via the circulation to shocked animals, on the LPS-

induced changes in the number of polymorphonuclear leukocytes (PMN) in the bronchoalveolar lavage (BAL) fluid. Animals were maintained in the hemorrhagic shock state for 60 min, followed by reperfusion with shed blood and an equal volume of Ringer's lactate with or without liposomal antioxidants, over a 2-h period. Following a period of 18 h after the initiation of shock, animals were challenged intratracheally with LPS (300 micrograms/kg body weight) and killed 4 h later. Each data point represents the mean ± SEM of 3 animals. The symbol "*" represents significantly different ($p<0.05$) from the corresponding value obtained from shocked animals treated with saline and challenged with LPS.

FIGS. 5A-B are photographs showing the results of a Northern blot assay. FIG. 5A shows expression of cytokine-induced neutrophil chemoattractant (CINC) mRNA, and FIG. 5 B shows expression of G3PDH mRNA (as a control). FIGS. 5C-D are bar graphs showing the effects of free N-acetyl cysteine (NAC), liposomal NAC (L-NAC), liposomal α-tocopherol (L-αT), or liposomal α-tocopherol and NAC (L-αT-NAC), administered to shocked animals, on the LPS-induced changes in cytokine-induced neutrophil chemoattractant (CINC) expression in lung tissue. Thirty minutes after reperfusion, animals were intratracheally instilled with saline or different antioxidant preparations (FIG. 5C). Alternatively, the saline or antioxidant preparations were administered via the circulation during the 2 hour reperfusion period (FIG. 5D). Animals were maintained in the hemorrhagic shock state for 60 min, followed by reperfusion with shed blood over a 2-h period. Thirty minutes after reperfusion, animals were intratracheally instilled with saline or different antioxidant preparations (Panel A). Alternatively, the saline or antioxidant preparations were administered via the circulation during the 2-h reperfusion period (Panel B). Following a period of 18 h after the initiation of shock, animals were challenged intratreacheally with LPS (300 micrograms/kg body weight) and 4 h later, animals were killed and their lung tissues harvested for the Northern blot procedure. Corresponding G3PDH mRNA bands (in lanes 1 - 6, each representing the corresponding treatment group shown in FIGS. 5C-D) are shown as evidence of comparable loading. Scanning densitometry of Northern blots for CINC mRNA was normalized by densitometry of corresponding G3PDH mRNA bands and expressed as mean ±SEM of 4 animals per group. The symbol "*" represents significantly different ($p<0.01$) from the corresponding value obtained from shocked

animals treated with saline and challenged with LPS.

FIGS. 6A-B are bar graphs showing the effects of free N-acetyl cysteine (NAC), liposomal NAC (L-NAC), liposomal α-tocopherol (L-αT), or liposomal α-tocopherol and NAC (L-αT-NAC), administered to shocked animals, on the LPS-induced changes in angiotensin converting enzyme (ACE) activity, an injury marker of pulmonary endothelial cells. Thirty minutes after reperfusion, animals were intratracheally instilled with saline or different antioxidant preparations (FIG. 6A). Alternatively, the saline or antioxidant preparations were administered via the circulation during the 2 hour reperfusion period (FIG. 6B). Animals were maintained in the hemorrhagic shock state for 60 min, followed by reperfusion with shed blood over a 2-h period. Thirty minutes after reperfusion, animals were intratracheally instilled with saline or different antioxidant preparations (FIG. 6A). Alternatively, the saline or antioxidant preparations were administered via the circulation during the 2-h reperfusion period (FIG. 6B). Following a period of 18 h after the initiation of shock, animals were challenged intratracheally with LPS (300 micrograms/kg body weight) and killed 4 h later. Each data point represents the mean ± SEM of 6 animals. The symbol "*" represents significantly different ($p<0.05$) from the corresponding value obtained from shocked animals treated with saline and challenged with LPS.

FIGS. 7A-B are bar graphs showing the effects of free N-acetyl cysteine (NAC), liposomal NAC (L-NAC), liposomal α-tocopherol (L-αT), or liposomal α-tocopherol and NAC (L-αT-NAC), administered intratracheally to the lungs of shocked animals, on the LPS-induced changes in transpulmonary albumin flux. Animals were maintained in the hemorrhagic shock state for 60 min, followed by reperfusion with shed blood over a 2-h period. Thirty minutes after reperfusion, animals were intratracheally instilled with saline or different antioxidant preparations. Following a period of 18 h after the initiation of shock, animals were challenged intratracheally with LPS (300 micrograms kg body weight) and 4 h later, their transpulmonary albumin flux was assessed as described in the text. Each data point represents the mean ± SEM of 3 animals in each group. The symbol "*" represents significantly different ($p<0.01$) from the corresponding value obtained from shocked animals treated with saline and challenged with LPS.

FIG. 8 is a bar graph showing the effects of free N-acetyl cysteine (NAC), liposomal NAC (L-NAC), liposomal α-tocopherol (L-αT), or liposomal α-tocopherol and NAC (L-αT-NAC), administered via the circulation to shocked animals, on the LPS-induced changes in the plasma levels of alanine aminotransferase (ALT), an indicator of liver damage. Animals were maintained in the hemorrhagic shock state for 60 min, followed by reperfusion with shed blood and an equal volume of Ringer's lactate with or without liposomal antioxidants, over a 2-h period. Following a period of 18 h after the initiation of shock, animals were challenged intratracheally with LPS (300 micrograms/kg body weight) and killed 4 h later. Each data point represents the mean ± SEM of 6 animals. The symbol "*" represents significantly different ($p<0.05$) from the corresponding value obtained from shocked animals treated with saline and challenged with LPS.

DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions are disclosed herein for the production of a therapeutic agent for the treatment of inflammatory complications associated with pulmonary and hepatic injuries, induced by hemorrhagic shock and endotoxemia. The compositions comprise bifunctional liposomal vesicles containing a hydrophilic sulfhydryl agent encapsulated in the aqueous interior of the vesicle and a lipophilic antioxidant incorporated in the vesicle membrane. One of the novel characteristic of the present invention resides in its sustained release property, which enables the initial delivery and subsequent retention of the active therapeutic agent(s) at the injured tissue "target site". For example, antioxidants are release shortly after administration (e.g, within 30 minutes) and continue to be released for a prolonged period of time, e.g., for 6 hours, 12 hours, 24 hours, and up to several days post-administration.

The compositions are prepared by producing liposomes with a specific combination of bilayer-forming lipids, which are compatible with, and non-toxic to, pulmonary tissues. A wide variety of lipids including, but not limited to, phosphatidyl esters and ethers (*e.g.*, phosphatidylcholine, phosphatidylethanolamine, etc.); glycerides; cerebrosides; sphingomyelin; gangliosides; steroids (*e.g.*, cholesterol); and the like, may be utilized in the production of the liposomes disclosed herein. One or more lipid entities may be present in the liposome, with a bilayer-forming lipid constituting the major liposomal component and the other lipid (*e.g.*, cholesterol) constituting the minor

component.

The biologically-active, therapeutic ingredients are incorporated in the liposomal microcapsules and do not interfere with the integrity, nor the stability of the lipid carrier. Moreover, the therapeutic agents comprising the liposomal formulations of the present invention may also serve to increase the overall structural and/or chemical stability of said formulations. Typically, the hydrophilic component of the liposomal preparation is comprised of a compound possessing a sulfhydryl group with free-radical scavenging and antioxidant properties. The hydrophobic therapeutic agent, also possessing strong antioxidant properties, is incorporated into the liposomal bilayer. The vesicles were prepared by combining the selected lipids, in appropriate ratios, in the presence of the lipophilic antioxidant, followed by the subsequent entrapment of the hydrophilic antioxidant. The procedure is conducted in such a manner so as not to denature, inactivate, or compromise the therapeutic efficacy of said antioxidants. Numerous methodologies may be utilized for liposome production, including, but not limited to, Shek, *et al.*, 1985. *Immunology* 57: 153-157, 1985; Jurima-Romet and Shek, 1991. *J. Pharm. Pharmacol.* 43: 6-10; Suntres and Shek, 1994. *J. Pharm. Pharmacol.* 46: 23-28, whose disclosures are incorporated herein by reference in their entirety.

The liposomal antioxidant formulation may be administered to a vertebrate host by acceptable conventional methods, including, but not limited to, intratracheal, intravenous, intraarterial, and intraperitoneal procedures. The administered dose will vary depending upon the specific antioxidant composition and the recipient. A therapeutic regimen can be established by determining the antioxidant retention time at the target body-site and the extent of local inflammation and injury. The liposomal antioxidant preparation may be used for the treatment of inflammatory complications associated with sepsis, trauma, and adult respiratory distress syndrome.

In contrast to the aforementioned results for NAC, the administration of liposome-entrapped α-tocopherol, alone, has been reported to significantly attenuate endotoxin-induced tissue injury in the liver and the lung. *See,* Suntres and Shek, 1996. *Shock* 6: S57-64, 1996; Suntres and Shek, 1996. *J. Endotoxin. Res.* 3: 505-512. Liposome-entrapped α-tocopherol also has been shown to reduce the toxic effects of reactive oxygen species released from phorbol myristate acetate-stimulated pulmonary target cells and infiltrating neutrophils. *See,* Suntres and Shek, 1995. *J. Drug Targeting.* 3: 201-208. The doses of NAC administered according to the invetion are at least 1-2 logs

less than the doses administered using previously described therapeutic methods. The methods described herein are therefore safer than earlier methods.

DETAILED DESCRIPTION OF THE INVENTION

I. Preparation of Liposome-Associated Anti-Oxidants

Liposome preparations consisted of either DPPC:cholesterol 7:3 with NAC entrapped; DPPC:α-tocopherol:cholesterol 7:2:1 with NAC entrapped; or DPPC:α-tocopherol:cholesterol 7:2:1 without NAC. The lipids were dissolved in chloroform:methanol (2:1, v/v) and the lipid solution was dried in a water-bath at 40°C under a stream of helium gas to a thin film, coating the interior surface of a round-bottomed glass vessel. Any residual solvent was removed by placing the vessel under vacuum for at least 18 hours. The dried lipid was then hydrated with either 1 ml of 200 mg/ml NAC for every 100 mg of lipid or 1 ml of phosphate-buffered saline for every 100 mg of lipid at 51°C. The glass vessel was vortexed periodically and kept at this temperature for one hour to form multilamellar vesicles. The multilamellar vesicles were subjected to a total of 5 freeze/thaw cycles using liquid nitrogen and a 40°C water-bath. The multilamellar vesicles were then extruded a total of 10-times with an extruder (Lipex Biomolecules; Vancouver, BC) fitted with two, stacked polycarbonate filters of various pore sizes (*e.g.*, 100 nm, 400 nm) under a helium pressure of 100 to 200 p.s.i. Non-entrapped NAC was removed by washing the liposomes twice in phosphate-buffered saline (PBS) and pelleting by centrifugation at 105,000 x *g* for 1 hour at 5°C in a Beckman L8-70 ultracentrifuge. Supernatant and resuspended pellet fractions were then assayed to determine overall NAC entrapment, and liposomal vesicle size was determined with the use of a Coulter N4SD particle size analyser. The final NAC liposome preparations were diluted to a concentration of 25.5 mg/ml before use.

Table 1 illustrates particle sizing and the entrapment efficiencies of the liposomal antioxidants of the present invention.

Table 1

Liposome Composition (molar ratio)	Vesicle Size (mean ± MEM)	Entrapment Efficiency N-acetyl cysteine	Entrapment Efficiency α-Tocopherol
DPPC:Chol (7:3)	337.3 ± 15.0	27.9 ± 4.5%	100%
DPPC: α-T:Chol (7:2:1)	477.0 ± 7.00	21.2 ± 0.5%	100%

Data represent Mean ± SEM of three experimental determinations.

Abbreviations: Chol = cholesterol; α-T = α-tocopherol

As shown in Table 1, α-tocopherol incorporated with a high degree of efficiency into the liposomal bilayers, with 100% entrapment at the lipid molar ratio used. The anti-oxidant, α-tocopherol is an extremely viscous and highly insoluble liquid, which renders it very difficult, if not impossible, to administer parenterally. The liposomal formulation disclosed herein provides a vehicle to facilitate the incorporation of α-tocopherol and its parenteral delivery. Additionally, the same liposome vehicle also enables the encapsulation of N-acetyl cysteine (NAC) for concomitant delivery of both anti-oxidants. N-acetyl cysteine was encapsulated in the vesicles at an entrapment efficiency of about 21-28%. Unlike previously described methods which describe liposomes which entrap a hydrophilic agent or those which entrap a lipophilic agent, the methods described herein co-entrap a hydrophilic and lipophilic agent such as tocopherol and NAC.

Preferred Liposomal Antioxidant Composition

For intratracheal administration:

Formula 1

Dipalmitoylphosphatidylcholine	7.56 mg
α-Tocopherol	1.27 mg
Cholesterol	0.57 mg
Isotonic saline	94 µl

Formula 2	
Dipalmitoylphosphatidylcholine	7.67 mg
Cholesterol	1.73 mg
N-acetylcysteine	3.76 mg
Isotonic saline	94 µl
Formula 3	
Dipalmitoylphosphatidylcholine	7.56 mg
α-Tocopherol	1.27 mg
Cholesterol	0.57 mg
N-acetylcysteine	3.76 mg
Isotonic saline	94 µl

For intravenous administration:

Formula 4	
Dipalmitoylphosphatidylcholine	37.82 mg
α-Tocopherol	6.34 mg
Cholesterol	2.85 mg
Isotonic saline	470 µl
Formula 5	
Dipalmitoylphosphatidylcholine	38.34 mg
Cholesterol	8.66 mg
N-acetylcysteine	18.8 mg
Isotonic saline	470 µl
Formula 6	
Dipalmitoylphosphatidylcholine	37.82 mg
α-Tocopherol	6.34 mg
Cholesterol	2.85 mg
N-acetylcysteine	18.8 mg
Isotonic saline	470 µl

Preferred Lipid and Antioxidant Molar Ratio

Formula 7	
Dipalmitoylphosphatidylcholine	7.00
Cholesterol	3.03
N-acetylcysteine	15.45

Formula 8

Dipalmitoylphosphatidylcholine	7.00
α-Tocopherol	1.97
Cholesterol	1.02
N-acetylcysteine	15.59

Preferred Liposome Size (prepared by the extrusion method)

a) For intratracheal administration:

Liposomes containing α-tocopherol and N-acetylcysteine 477 ± 7.0 nm

b) For intravenous administration:

Liposomes containing α-tocopherol and N-acetylcysteine 149 ± 0.3 nm

Preferred Liposomal Antioxidant Dosage for Effective Therapy

a) For intratracheal administration:

N-acetylcysteine in α-tocopherol liposomes 9.4 mg/kg body weight

b) For intravenous administration:

N-acetylcysteine in α-tocopherol liposomes 47.0 mg/kg body weight

II. Prolonged Anti-Oxidant Retention in the Lung

The therapeutic efficacy of an antioxidant in treating oxidant stress-induced lung injury, to a large extent, depends upon the availability of the antioxidant in sufficient quantities in the pulmonary milieu. Most, if not all of the published studies have disclosed very limited (*e.g.*, 0.5-5% of initial dose) pulmonary uptake of α-tocopherol following intragastric and parenteral administration. In contrast, the results disclosed herein demonstrate that the intratracheal administration of liposomal α-tocopherol resulted in a total pulmonary level of about 1.5 mg or a retention of about 79% of the administered α-tocopherol dose at 24 hours post-administration (*see,* FIG. 1A).

The retention of NAC within the lungs of normal rats treated intratracheally with free NAC; liposomal NAC; or α-tocopherol liposomal NAC is shown in FIG. 1B. Recovery of NAC in the lung was approximately 1% of the initial dose 1 hour after the administration of free NAC, and subsequently declined to 0.2% of initial dose approximately 3 hours, later. In direct contrast, the recovery of NAC after the administration of L-NAC was found to be approximately 8% and 3% of initial dose at 1 hour and 24 hours post-administration, respectively. The pulmonary retention of NAC

following the administration of α-tocopherol liposomal NAC followed a similar retention characteristics to that of liposomal NAC, but it was lower.

III. Animal Model of Hemorrhagic Shock and Lung Injury

Male Sprague-Dawley rats (300-350 grams in weight) were anesthetized with intraperitoneally administration of ketamine (80 mg/kg) and xylazine (8 mg/kg). The right carotid artery was cannulated with a 22-gauge angiocath (Becton Dickinson; Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling and resuscitation. Hemorrhagic shock was then initiated by blood withdrawal and reduction of the MAP to 40 mm Hg within 15 minutes. This blood pressure was subsequently maintained by further blood withdrawal if the MAP rose to a level of greater-than 45 mm Hg, and by infusion of 0.5 ml of Ringer's Lactate (RL) if the MAP dropped to a level greater-than 35 mm Hg. Withdrawn blood was collected into a solution of 0.1 ml citrate/ml of blood, to prevent clotting. After a hypotensive period of 60 minutes, animals were resuscitated by transfusion of the withdrawn blood and RL in a volume equal to that of withdrawn blood, over a period of two hours. The catheter was then removed, the carotid artery ligated, and the cervical incision sutured. Control (sham) animals underwent the same surgical procedures, but hemorrhage was not induced. NAC delivery occurred in the control animals at an equivalent time to that received by the experimental animals in which shock was induced.

Thirty minutes after resuscitation, the endotracheal intubation of liposome-associated NAC and/or α-tocopherol was performed. The animal was placed on a slanted board (20° from the vertical) and was supported by an elastic band under its' upper incisors. A microscope lamp, with its beam directed at the neck area, provided transillumination during the procedure. By opening the mouth of the animal and depressing the tongue, the larynx could be easily visualized. The liposomal suspension was delivered to the lung, via the intratracheal administration, using PE-50 polythylene tubing (6.5 cm) connected to a 25-gauge epidural catheter. The endotracheal tube was introduced into the trachea using gentle pressure. All animals received 150 µl of a liposomal preparation which contained 9.4 mg/kg body weight of NAC and/or α-tocopherol, followed by 20 mechanically-ventilated breaths using a rodent ventilator. Eighteen hours after hemorrhage-resuscitation, lipopolysaccharide (LPS; *Escherichia coli* strain O11B4; at a concentration of 300 µg/kg in 200 ml saline) was administered

intratrachealy. Animals were sacrificed in 4-6 hours by a pentobarbital overdose.

IV. Lung Tissue Preparation

The lungs were removed from animals immediately after decapitation and rinsed with ice-cold saline to remove residual blood. All subsequent steps were carried out at 0-4°C. 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.

V. Lipid Peroxidation Determination

Lipid peroxidation products in lung homogenates (*i.e.*, malonaldehyde (MDA) and 4-hydroxyalkenals (4-HNE)), were measured by the use of an assay kit (R&D Systems; Minneapolis, MN). This assay is based upon the reaction of a chromogenic reagent (N-methyl-2-phenylindole), with MDA and 4-HNE at 45°C. One molecule of either MDA or 4-HNE reacts with 2 molecules of the chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm. The concentration of MDA and 4-HNE is then quantitated by the absorbance at this wavelength.

VI. Determination of Pulmonary Non-Protein Sulphydryl Concentration

The non-protein sulphydryl concentration, which includes glutathione and NAC, in pulmonary homogenates was determined as described by Suntres and Shek (1994. *J. Pharm. Pharmacol.* 46: 23-28). Briefly, the tissue was homogenized in 20% (w/v) trichloroacetic acid and centrifuged at 600 x g for 20 minutes in a refrigerated Beckman GS-6R centrifuge. An aliquot of the deproteinized supernatant fraction was added to 2 ml of 0.3 M Na_2HPO_4 solution followed by addition of 0.5 ml of 0.04% 5,5-dithiobis-[2-nitrobenzoic acid] (NbS_2) dissolved in 10% sodium citrate. The absorbance at 412 nm was measured immediately after mixing.

VII. Calculation of Pulmonary NAC Content

Since the assay performed above measures the total non-protein sulphydryl content (GSH + NAC) in the lungs of normal animals, the NAC values were obtained by subtracting the values for GSH (460 μg/lung) from the total non-protein sulfhydryl values.

VIII. Enzyme Measurements

The activity of angiotensin converting enzyme (ACE) in lung homogenates was determined by using a kit (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's protocol. The activity of myeloperoxidase (MPO) in sonicated whole lung homogenates was performed using an assay kit (R&D Systems; Minneapolis, MN) according to the manufacturer's directions. Plasma alanine aminotransferase (ALT) activity, expressed as Sigma Frankel (SF) units/ml, was determined with a diagnostic kit (No. 505; Sigma Chemical Company; St. Louis, MO).

IX. Reduction of Pulmonary Oxidant-Stress After Treatment with Liposomal Anti-Oxidants

Pulmonary Lipid Peroxidation:

The level of lipid peroxidation has been used as an indicator of oxidative stress. Challenge of shocked with LPS produced a significant increase in lipid peroxidation in pulmonary homogenates (*i.e.*, an 18-fold increase), as measured by the formation of MDA and 4-HNE. Pre-treatment of rats with NAC did not significantly alter the LPS-induced increases in lipid peroxidation (*see,* FIG. 2A). Conversely, pre-treatment of rats with NAC- or α-T-containing liposomes or liposomes containing both α-T and NAC, were found to partially protect against the LPS-induced lipid peroxidation at levels of 55%, 38%, and 62%, respectively.

Pulmonary Non-Protein Thiols:

As the non-protein sulphydryls (NP-SH), glutathione and NAC, are known to play an important role in protecting cells against oxidant-induced tissue injury, the concentration of non-protein sulphydryls in lung tissues were also measured. The administration of LPS in shocked animals was found to result in a significant reduction (43%) in NP-SH concentration (*see,* FIG. 2B). Intratracheal administration of NAC did not significantly increase the NP-SH content of the lung.

In contrast, intratracheal administration instillation of liposomes containing NAC or NAC and α-tocopherol was shown to result in a significant increase in the pulmonary NP-SH content, which may be attributed to the retention of NAC within the lung.

X. Bronchoalveolar Lavage Preparation

For Bronchoalveolar Lavage Preparation (BAL), the lungs were lavaged with cold

phosphate-buffered saline (PBS; 8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, pH 7.4 with 0.1 mM EDTA) using an intratracheal angiocath. The PBS was instilled in 10 ml aliquots, and gently withdrawn with a 10 ml syringe, so as to provide a total administered volume of 40 ml. The collected BAL fluid was then centrifuged at 300 x *g* for 10 minutes to pellet cells. The supernatant was discarded, and the pelleted cells were resuspended in a small volume of serum-free DMEM culture medium (Gibco; Burlington, Ontario). Total cell counts were determined on a grid hemocytometer. Differential cell counts were enumerated on cytospin-prepared slides that were stained with Wright-Giemsa stain. A total of 500 cells were counted in cross-section per sample and the number of polymorphonuclear leukocytes (PMN) and alveolar macrophages were calculated as the total cell count multiplied by the percentage of the respective cell type in the BAL fluid (BALF) sample.

XI. Quantitation of CINC mRNA Expression by Northern Blot Analysis

Total RNA from lungs was obtained using the guanidium-isothiocyanate method. *See,* Chomczynski and Sacchi, 1987. *Anal. Biochem.* 162: 156-160. In brief, the lungs were harvested from treated animals and immediately frozen in liquid nitrogen. The lungs were then thawed and homogenized in 10 ml of 4 M guanidine-isothiocyanate containing 25 mM sodium citrate, 0.5% sarcosyl, and 100 mM β-mercaptoethanol. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to nylon membrane. Hybridization was carried out using a [^{32}P]ATP-end-labeled 30-base oligonucleotide probe for the cytokine-induced neutrophil chemoattractant (CINC) possessing the with the nucleotide sequence

5'-GCGGCATCACCTTCAAACTCTGGATGTTCT-3', [SEQ ID NO:1] which is complementary to nucleotides 134 to 164 of CINC cDNA (*see,* Balckwell, *et al.*, 1994. *Am. J. Respir. Cell Mol. Biol.* 11: 464-472), kindly provided by Dr. Timothy S. Blackwell; Vanderbilt University School of Medicine, Nashville, TN. Blots were then washed under conditions of high stringency and specific mRNA bands were detected by autoradiography in the presence of intensifying screens as previously reported. Blots were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), which is a ubiquitously expressed housekeeping gene to control for loading (*see,* Tso, *et al., Nucl. Acids Res.* 13: 2485-2490). Expression of mRNA was quantitated using a

phosphoimager and accompanying ImageQuant software (Molecular Dynamics; Sunnyvale, CA) and was normalized to the G3PDH signal.

XII. Reduction of Neutrophil Infiltration in the Lung After Treatment with Liposomal Anti-Oxidants

Lung Myeloperoxidase Activity:

Lung injury in shocked animals subsequently challenged with LPS, is generally associated with the infiltration and activation of neutrophils. This neutrophilic infiltration in the lungs of shocked animals challenged with LPS, was assessed by measuring the activity of myeloperoxidase (MPO), an enzyme localized primarily in neutrophils. As shown in FIG. 3A, the MPO activity in shocked animals was increased by 16-fold, following LPS administration. This increase is suggestive of neutrophil infiltration within the lungs. A very similar increase in MPO activity was also observed in LPS-challenged animals pretreated with NAC. Although L-αT liposomal treatment prevented some neutrophil infiltration (*i.e.*, 16% reduction), L-NAC and L-αT-NAC had a more pronounced suppressive effect against neutrophil infiltration in the lung, with a 30% and 35% reduction, respectively.

Neutrophil Infiltration:

Hemorrhage-resuscitation followed by LPS administration caused a 14-fold increase in polymorphonuclear leukocyte (PMN) infiltration, in comparison to that in the control (sham) animals (*see*, FIG. 3B). The increase in PMN in the shock/LPS animal group was attenuated to 71.2%, 80.0%, and 58.9% by liposome-associated NAC; liposome-associated α-tocopherol, and liposome-associated NAC/α-tocopherol, respectively. Empty liposome alone, did not alter PMN infiltration. Similarly, intratracheal administration of NAC alone, did not decrease PMN influx after shock/LPS administration. A very similar pattern of liposomal antioxidant-mediated reduction in PMN infiltration was also evident upon anti-oxidant administration via the circulation (*see*, FIG. 4).

CINC mRNA Expression:

In order to determine whether the alteration in polymorphonuclear leukocyte (PMN) infiltration is associated with changes in cytokine-induced neutrophil

chemoattractant (CINC) expression, total RNA was extracted from whole lung tissue 4 hours after LPS administration. Northern blot analysis for CINC mRNA was then performed.

As shown in FIGS. 5A-D, it was determined that antecedent shock primed the increase in CINC mRNA in response to a subsequent LPS challenge. However, the administration of liposome-associated NAC with or without α-tocopherol via the trachea (*see*, FIG. 5C) or the circulation (*see*, FIG. 5D), significantly decreased the CINC mRNA expression, in comparison to those mRNA expression levels found in shocked animals treated with saline and challenged with LPS. Since the inventors of the present invention have previously shown that CINC is the major chemokine, which contributes to PMN influx into alveoli in a two-hit model (*see*, Fan, *et al.*, 1998. *J. Immunol.* 161: 440-447), the sustained effect of NAC on preventing PMN infiltration may be mediated by a decrease in CINC expression.

XIII. Assessment of Transpulmonary Albumin Flux

Transpulmonary albumin flux was assessed by injecting 1 mCi of ^{125}I-albumin, in a total volume of 0.2 ml saline, into the tail vein of the rat immediately following intratracheal administration of LPS or saline (*see*, Nathens, *et al.*, 1996. *Surgery* 120: 360-366). Six hours after LPS administration, 1 ml of blood was withdrawn by cardiac puncture for scintillation counting by the following procedure. Following exsanguination, the lungs were perfused via a cannula *in situ* with 10 ml of PBS. The perfused PBS was withdrawn gently and a volume of 1 ml/tube was aliquoted for counting. The transpulmonary albumin flux was normalized to blood cpm using the following formula:

$$\text{Transpulmonary Albumin Flux} = \frac{\text{BALF cpm/ml}}{\text{Blood cpm/ml}}$$

XIV. Evidence of Reduced Lung Damage by Treatment with Liposomal Anti-Oxidants

Lung Angiotensin Converting Enzyme:

Due to the fact that angiotensin converting enzyme (ACE) has been used as an injury marker of pulmonary endothelial cells, the effect of LPS on the activity of this

enzyme in lung homogenates of shocked animals pre-treated with saline, NAC, L-NAC, Lα-T, or L-αT-NAC, was measured. As shown in FIGS. 6A-B, the challenge of shocked animals with LPS produced a significant reduction in ACE (35%) in lung homogenates of saline-pretreated animals. Treatment of animals with NAC failed to attenuate the LPS-induced decreases in ACE activity, whereas treatment of animals with NAC-containing liposomes conferred a protective effect (19% of saline-pre-treated animals). Additionally, pre-treatment of animals with L-αT or L-αT-NAC also ameliorated the LPS-induced changes in ACE activity, to approximately the same level as that which was observed following L-NAC treatment. The administration of the liposomal preparations via the tracheal (*see*, FIG. 6A) or the circulation (*see*, FIG. 6B) were effective in maintaining ACE activities in the lung, and therefore in reducing the extent of associated pulmonary endothelial cell damage.

Transpulmonary Albumin Flux:

In order to evaluate whether liposome-associated NAC and/or α-tocopherol could prevent lung injury, transpulmonary albumin flux was meausred 24 hours after hemorrhage-resuscitation and 6 hours after intratracheal administration of LPS. As shown in FIGS. 7A-B, the antecedent shock and the subsequent challenge with LPS, markedly increased lung permeability index (PI). The intratracheal administration of liposome-associated NAC, α-tocopherol, and NAC/α-tocopherol significantly attenuated the increase in PI to 27.7%, 50.9%, and 20.4%, respectively, as compared to that of shocked animals treated with saline and challenged with LPS.

XV. Reduced Hepatic Damage in Animals Treated with Liposomal Anti-Oxidants

Plasma Alanine Aminotransferase (ALT) Enzyme:

The measurement of hepatic enzymes such as plasma alanine aminotransferase (ALT) released into the blood has been shown to be a reliable indicator of hepatic injury. Plasma ALT activities were found to be elevated by greater than 8-fold in shocked animals subsequently challenged with LPS, thus indicating a rather substantial hepatic injury (*see*, FIG. 8). The administration of free antioxidant reduced the ALT down to about 6-fold, but the most effective treatment was mediated by the administration of liposomal antioxidants, which essentially prevented hepatic injury as indicated by the

presence of normal plasma ALT activities.

It is evident from the above observations that the said bifunctional liposome formulation containing both α-tocopherol and N-acetyl cysteine is effective in providing a therapeutic benefit for treating lung and liver injuries. Furthermore, the concept of bifunctional liposomes can be further exploited to coentrap other pairs of related antioxidants in therapeutic applications.

Equivalents

From the foregoing detailed description of the specific embodiments of the present invention, it should be readily apparent that a unique compositions and methods of treatment involving the use of uni- and multi-lamellar liposomes as a vehicle to provide systemic delivery of an antioxidant, via administration to the pulmonary system, have been disclosed. Although particular embodiments have been disclosed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims which follow. In particular, it is contemplated by the inventor that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims.

Other embodiments are within the following claims.

WHAT IS CLAIMED IS:

1. A liposomal composition comprising hydrophilic sulfhydryl agent and a lipophilic antioxidant, wherein said composition comprises at least 1% by weight of said hydrophilic agent.

2. The composition of claim 1, wherein said composition comprises free radical scavenging activity and antioxidant activity.

3. The composition of claim 1, wherein said composition comprises at least 10% by weight of said hydrophilic sulfhydryl agent.

4. The composition of claim 1, wherein said composition comprises at least 20% by weight of said hydrophilic sulfhydryl agent.

5. The composition of claim 1, wherein said composition comprises at least 25% by weight of said hydrophilic sulfhydryl agent.

6. The composition of claim 1, wherein said composition comprises at least 28% by weight of said hydrophilic sulfhydryl agent.

7. The composition of claim 1, wherein said hydrophilic sulfhydryl agent is encapsulated in an aqueous interior of a liposomal vesicle and said lipophilic antioxidant is incorporated an outer membrane of said liposomal vesicle.

8. The composition of claim 1, wherein said hydrophilic sulfhydryl agent is an antioxidant.

9. The composition of claim 8, wherein said antioxidant is N-acetyl cysteine.

10. The composition of claim 1, wherein said composition comprises at least 1% by weight of said lipophilic antioxidant.

11. The composition of claim 1, wherein said composition comprises at least 5% by weight of said lipophilic antioxidant.

12. The composition of claim 1, wherein said composition comprises at least 7% by weight of said lipophilic antioxidant.

13. The composition of claim 1, wherein said composition comprises at least 9% by weight of said lipophilic antioxidant.

14. The composition of claim 1, wherein said lipophilic antioxidant is α-tocopherol.

15. The composition of claim 1, wherein the range of size of liposomes in said composition is within 25% of the mean size of said liposomes.

16. The composition of claim 1, wherein the size of liposomes in said composition is between 100-400 nm.

17. The composition of claim 1, wherein the size of liposomes in said composition is uniform.

18. The composition of claim 1, wherein said composition comprises a phospholipid selected from the group consisting of a phosphatidylcholine, a dipalmitoylphosphatidylcholine, a lysophosphatidylcholine, a phosphatidylserine, a phosphatidyl-ethanolamine, a phosphatidylglycerol, and a phosphatidylinositol.

19. The composition of claim 18, wherein said composition further comprises cholesterol.

20. The composition of claim 1, wherein said composition does not comprise a metal.

21. The composition of claim 1, wherein said composition substantially lacks Zn, Se, Cr, Cu, or Mn.

22. A liposomal composition comprising hydrophilic sulfhydryl agent, a phospholipid, and cholesterol, wherein the approximate molar ratio of phospholipid:cholesterol is selected from the group consisting of 7:3, 6:4, and 9:1.

23. The liposomal composition of claim 22, wherein said phospholipid is dipalmitoylphosphatyidylcholine and wherein the approximate molar ratio of dipalmitoylphosphatyidylcholine:cholesterol is 7:3.

24. The liposomal composition of claim 21, wherein the approximate molar ratio of phospholipid:cholesterol:hydrophilic sulfhydryl agent is 7:3:15.

25. A liposomal composition comprising hydrophilic sulfhydryl agent, a phospholipd, cholesterol, and a lipophilic antioxidant, wherein the approximate molar ratio of phospholipid:lipophilic antioxidant:cholesterol:hydrophilic sulfhydryl agent is 7:2:1:15.

26. A method of delivering an antioxidant to a vertebrate comprising contacting a pulmonary tissue of said vertebrate with a liposomal composition comprising hydrophilic sulfhydryl agent and a lipophilic antioxidant, wherein said composition comprises at least 1% by weight of said hydrophilic agent.

27. The method of claim 26, wherein said vertebrate is a mammal.

28. The method of claim 26, wherein said vertebrate is a human.

29. The method of claim 26, wherein said vertebrate is identified as suffering from or at risk of developing a pulmonary injury, a hepatic injury, hemorrhagic shock, endotoxic insult, reperfusion injury, or adult respiratory distress syndrome.

30. The method of claim 26, wherein said composition is administered to said vertebrate by a intratracheal, intravenous, intraarterial, intraperitoneal, or intratissue route.

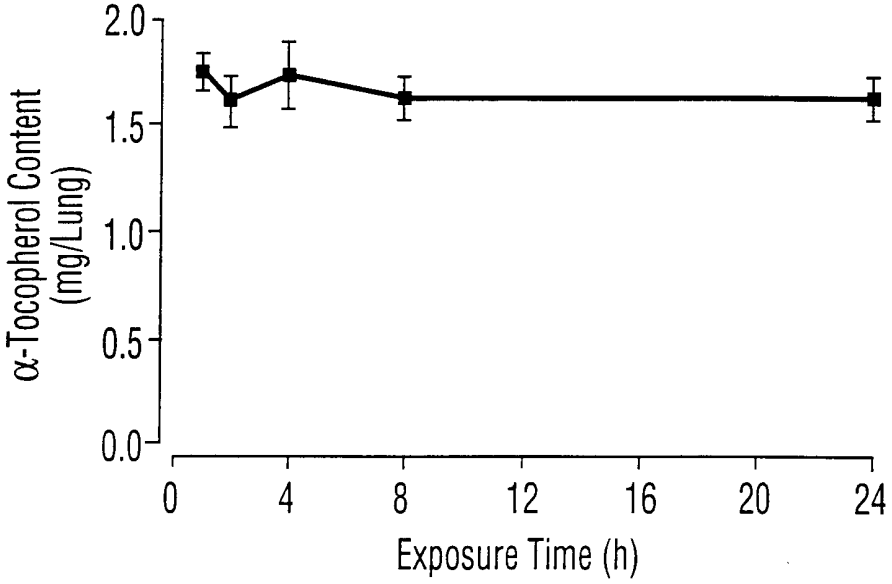

α-Tocopherol Content (mg/Lung)
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
Exposure Time (h)

FIG.1A

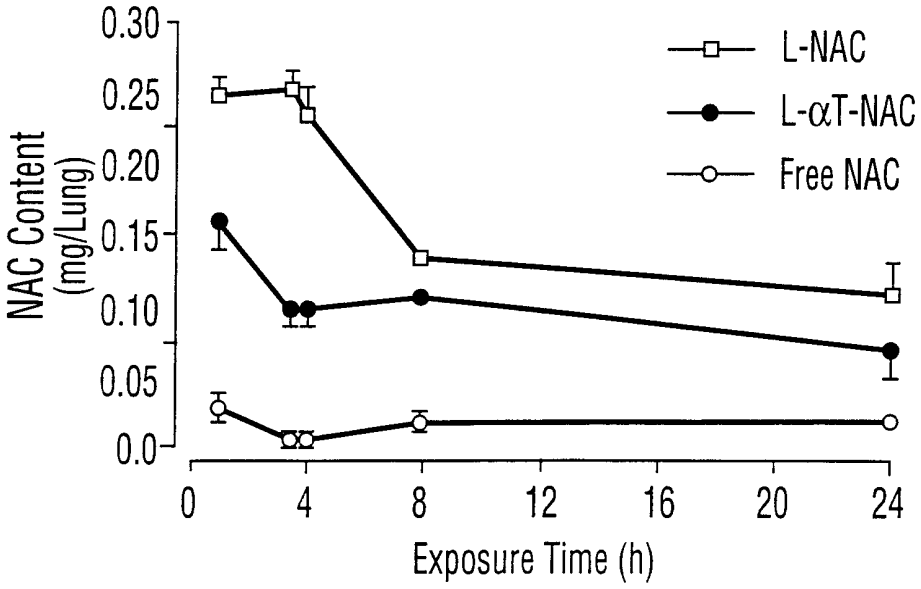

NAC Content (mg/Lung)
0.30
0.25
0.20
0.15
0.10
0.05
0.0
L-NAC
L-αT-NAC
Free NAC
0
4
8
12
16
20
24
Exposure Time (h)

FIG.1B

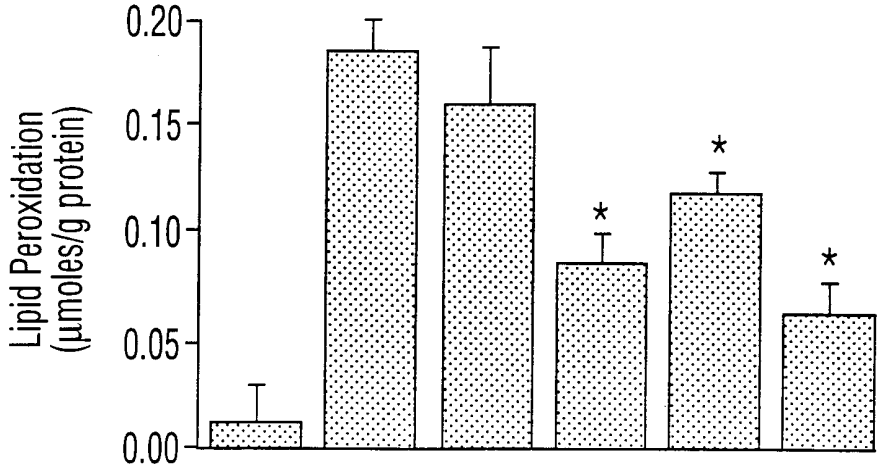

Lipid Peroxidation (µmoles/g protein)
0.20
0.15
0.10
0.05
0.00
*
*
*

FIG. 2A

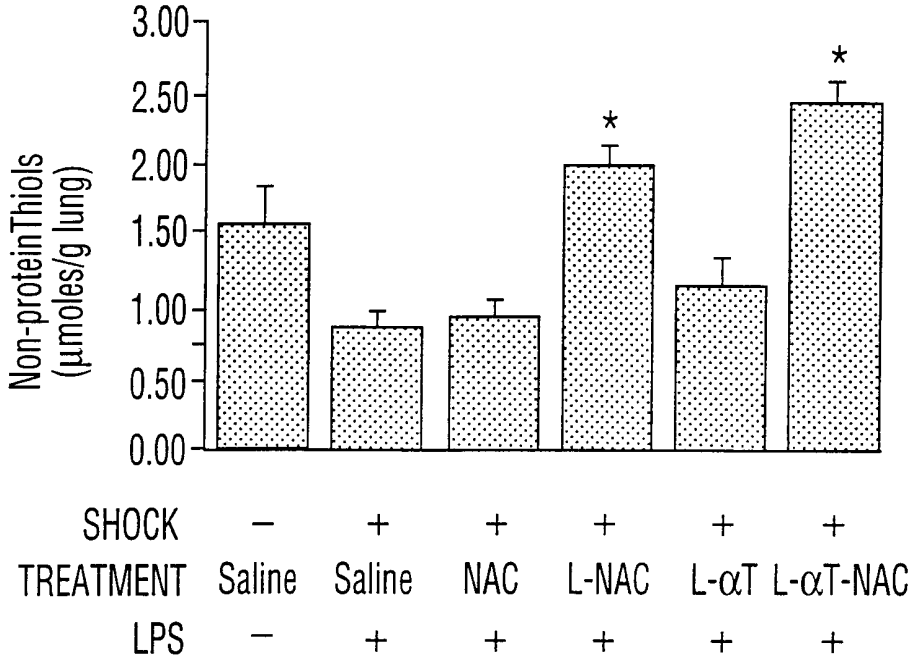

Non-proteinThiols (µmoles/g lung)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
*
SHOCK – + + + + +
TREATMENT Saline Saline NAC L-NAC L-αT L-αT-NAC
LPS – + + + + +

FIG. 2B

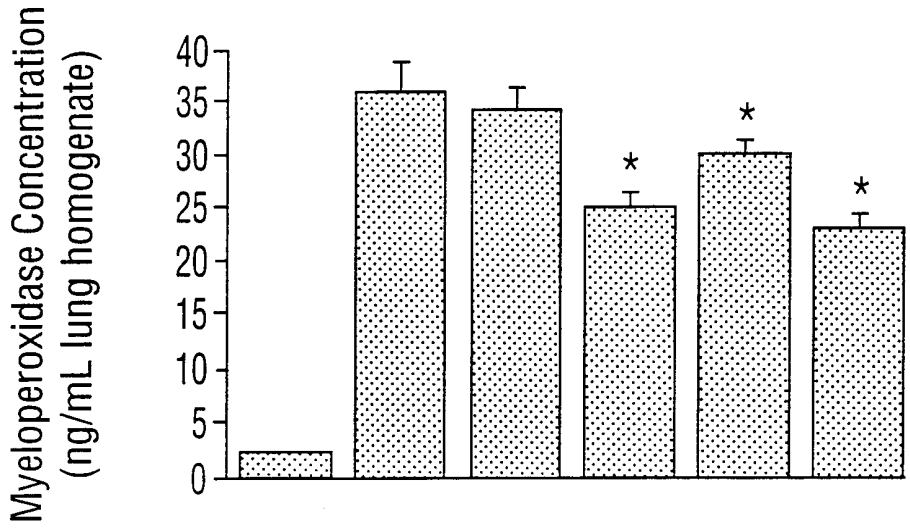

Myeloperoxidase Concentration
(ng/mL lung homogenate)
40
35
30
25
20
15
10
5
0
*
*
*

FIG. 3A

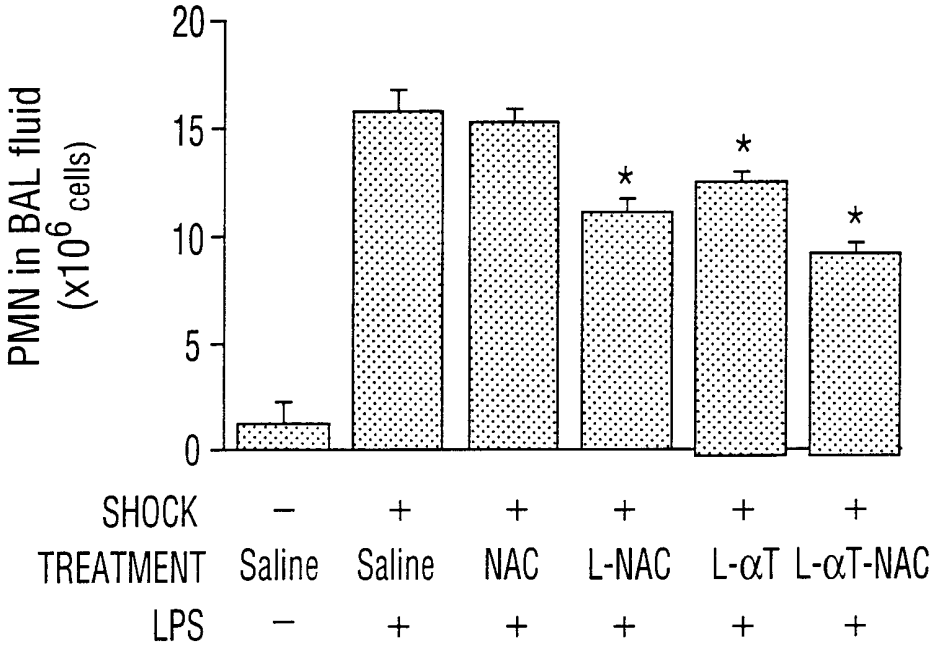

PMN in BAL fluid
(x10^6 cells)
20
15
10
5
0
*
*
*
SHOCK – + + + + +
TREATMENT Saline Saline NAC L-NAC L-αT L-αT-NAC
LPS – + + + + +

FIG. 3B

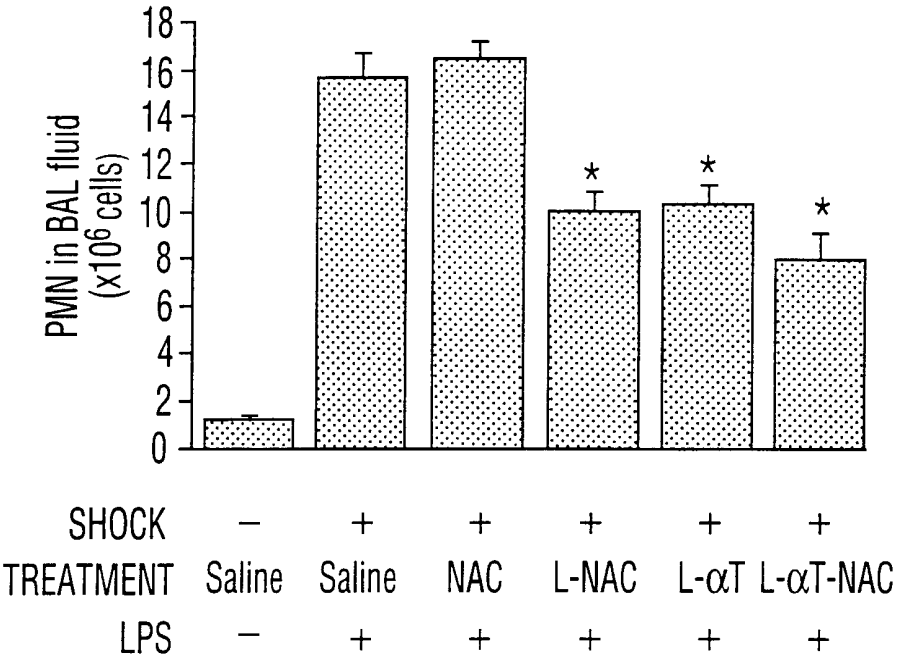

PMN in BAL fluid
(x10⁶ cells)
18
16
14
12
10
8
6
4
2
0
*
*
*
SHOCK – + + + + +
TREATMENT Saline Saline NAC L-NAC L-αT L-αT-NAC
LPS – + + + + +

FIG. 4

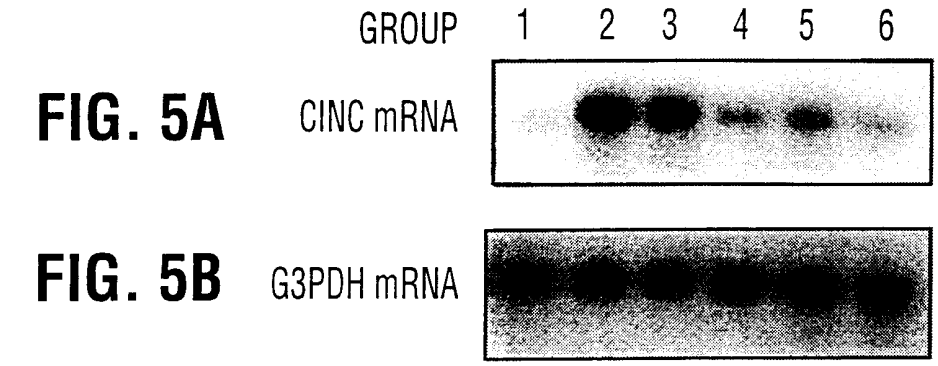


FIG. 5A

FIG. 5B

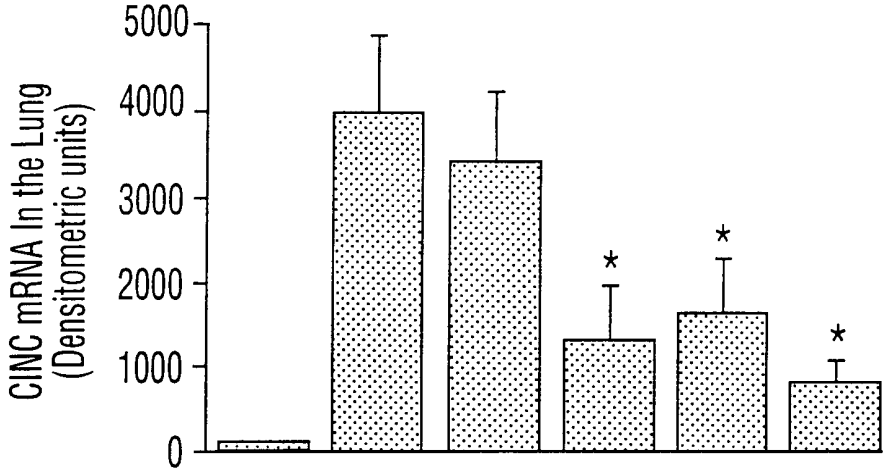


FIG. 5C

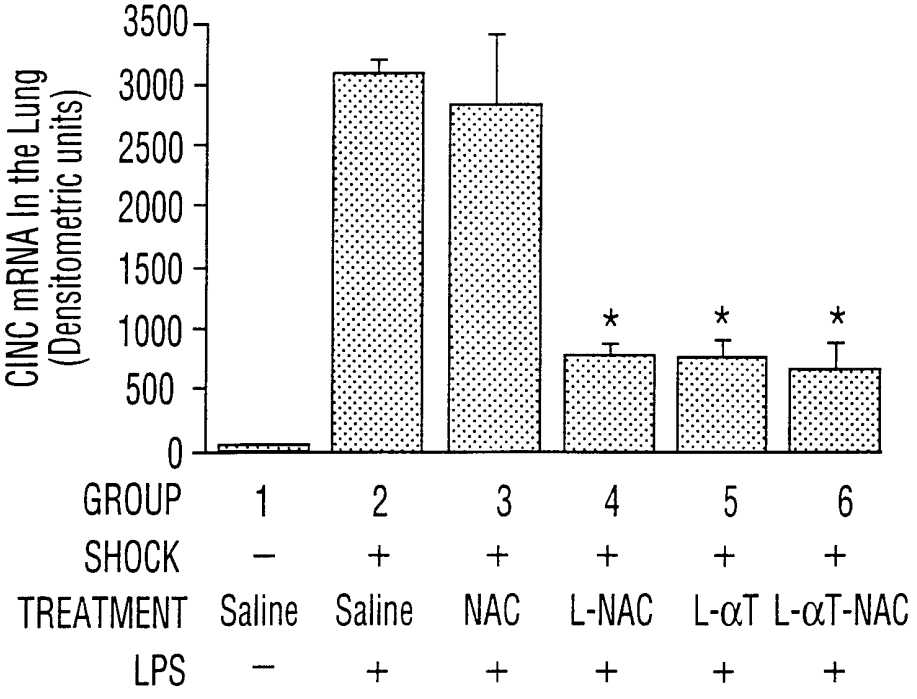


FIG. 5D

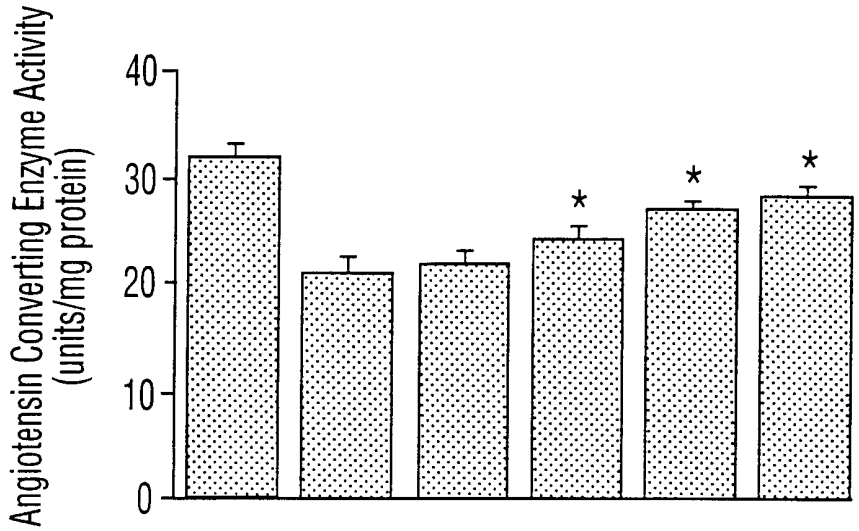

Angiotensin Converting Enzyme Activity
(units/mg protein)
40
30
20
10
0
*
*
*

FIG. 6A

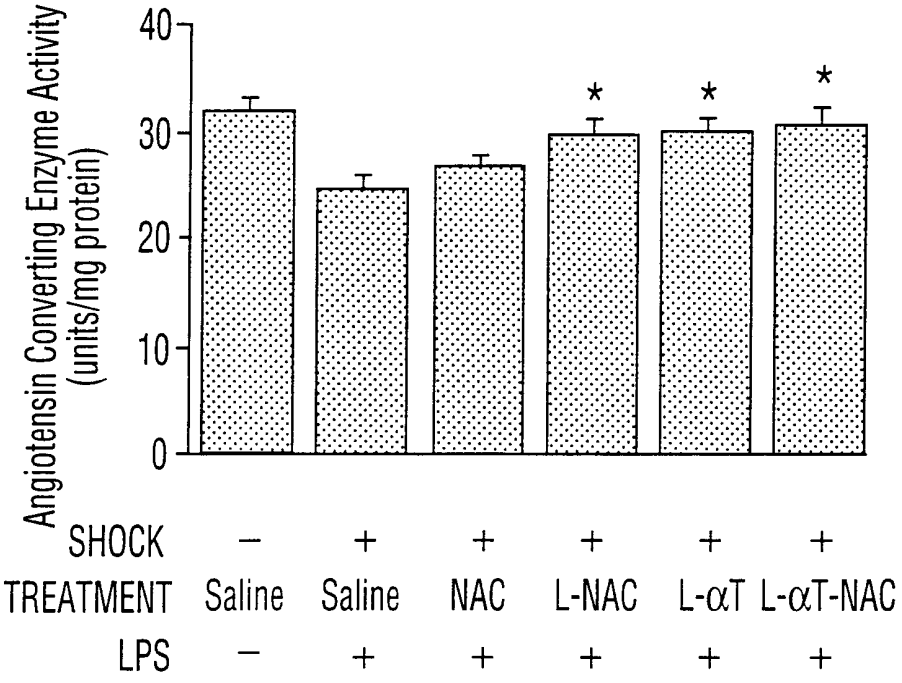

Angiotensin Converting Enzyme Activity
(units/mg protein)
40
30
20
10
0
*
*
*
SHOCK – + + + + +
TREATMENT Saline Saline NAC L-NAC L-αT L-αT-NAC
LPS – + + + + +

FIG. 6B

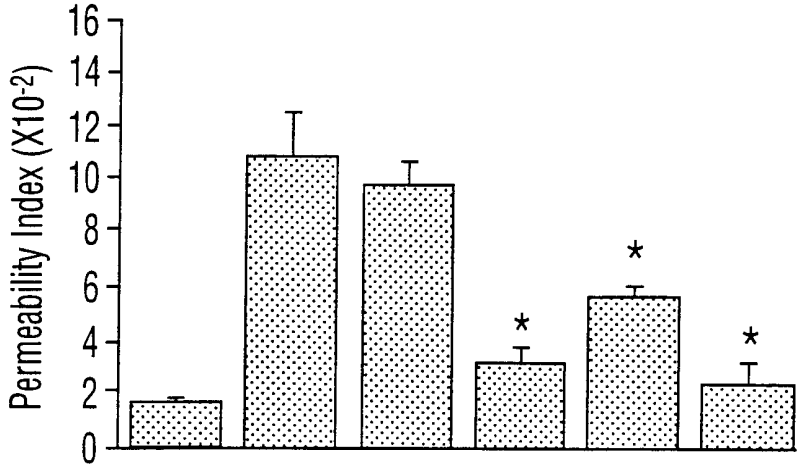

Permeability Index (X10-2)
16
14
12
10
8
6
4
2
0
*
*
*

FIG. 7A

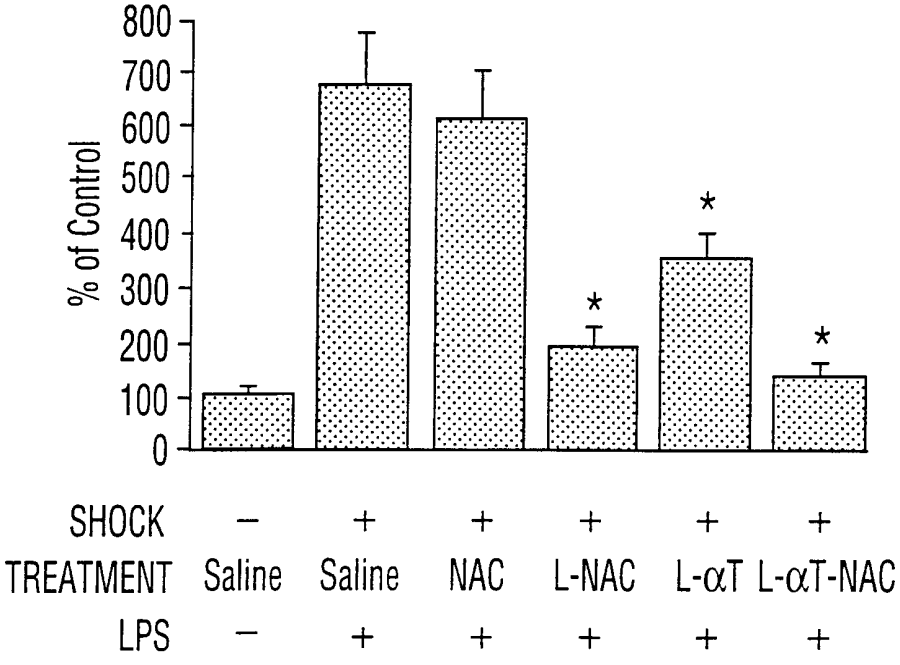

% of Control
800
700
600
500
400
300
200
100
0
*
*
*
SHOCK – + + + + +
TREATMENT Saline Saline NAC L-NAC L-αT L-αT-NAC
LPS – + + + + +

FIG. 7B

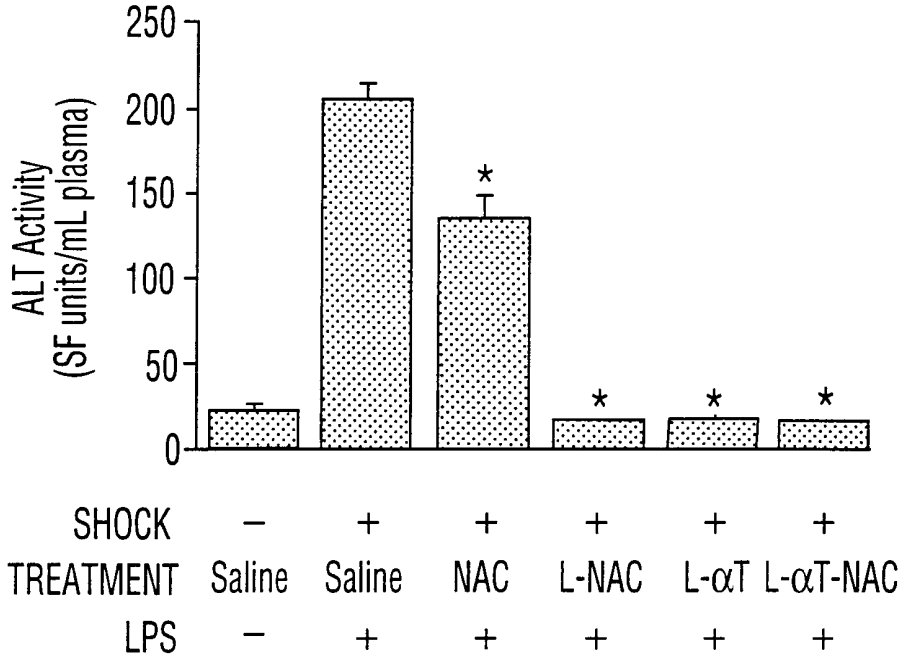

ALT Activity
(SF units/mL plasma)
250
200
150
100
50
0
*
*
*
*
SHOCK – + + + + +
TREATMENT Saline Saline NAC L-NAC L-αT L-αT-NAC
LPS – + + + + +

FIG. 8