International Journal of Pharmaceutics 363 (2008) 106–111

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Effectiveness of liposomal-*N*-acetylcysteine against LPS-induced lung injuries in rodents

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ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 11 July 2008
Accepted 15 July 2008
Available online 23 July 2008

Keywords:
Liposomes
N-Acetylcysteine
Oxidative stress
Inflammation
Antioxidants
Lung

ABSTRACT

Acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome (ARDS) are frequent complications in critically ill patients and are responsible for significant morbidity and mortality. So far, experimental evidence supports the role of oxidants and oxidative injury in the pathogenesis of ALI/ARDS. In this study, the antioxidant effects of conventional *N*-acetylcysteine (NAC) and liposomally entrapped *N*-acetylcysteine (L-NAC) were evaluated in experimental animals challenged with lipopolysaccharide (LPS). Rats were pretreated with empty liposomes, NAC, or L-NAC (25 mg/kg body weight, iv); 4 h later were challenged with LPS (*E. coli*, LPS 0111:B4) and sacrificed 20 h later. Challenge of saline (SAL)-pretreated animals with LPS resulted in lung injury as evidenced by increases in wet lung weight (edema), increases in lipid peroxidation (marker of oxidative stress), decreases of lung angiotensin-converting enzyme (ACE) (injury marker for pulmonary endothelial cells) and increases in the pro-inflammatory eicosanoids, thromboxane B_2 and leukotriene B_4. The LPS challenge also increased pulmonary myeloperoxidase activity and chloramine concentrations indicative of neutrophil infiltration and activation of the inflammatory response. Pretreatment of animals with L-NAC resulted in significant increases in the levels of non-protein thiols and NAC levels in lung homogenates ($p < 0.05$) and bronchoalveolar lavage fluids ($p < 0.001$), respectively. L-NAC was significantly ($p < 0.05$) more effective than NAC or empty liposomes in attenuating the LPS-induced lung injuries as indicated by the aforementioned injury markers. Our results suggested that the delivery of NAC as a liposomal formulation improved its prophylactic effectiveness against LPS-induced lung injuries.

1. Introduction

Acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome (ARDS), remain the leading factors of morbidity and mortality in critically ill patients (Costa et al., 2006; Frutos-Vivar et al., 2006). Sepsis is one of the most important causes of ARDS and it is estimated that 18–42% of patients with Gram-negative infections will develop ARDS with a mortality rate of approximately 50% (Bhatia and Moochhala, 2004; Costa et al., 2006). ARDS is an acute, severe injury to most or all of both lungs and it is characterized by severe hypoxemia, high-permeability pulmonary edema, neutrophil accumulation in the lung, reduction in lung compliance and increase in pulmonary resistance (Bhatia and Moochhala, 2004; Costa et al., 2006; Frutos-Vivar et al., 2006). The morphological manifestations may include damage to endothelial and epithelial sites of the blood-gas barrier (Bhatia and Moochhala, 2004; Costa et al., 2006; Frutos-Vivar et al., 2006). There is no specific therapy and the primary treatment involves supportive care in an intensive care unit (ICU), including use of a mechanical ventilator and supplemental oxygen (Bhatia and Moochhala, 2004; Wheeler and Bernard, 2007).

There is convincing evidence that reactive oxygen species (ROS) play a major role in mediating injury to the endothelial barrier of the lung in the presence of endotoxin or bacterial sepsis (Metnitz et al., 1999; Zhang et al., 2000; Bhatia and Moochhala, 2004). These toxic oxygen species are produced by activated neutrophils, macrophages, and stimulated pulmonary endothelial cells. ROS are capable of reacting with cellular lipids, proteins, nucleic acids

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doi:10.1016/j.ijpharm.2008.07.015

and carbohydrates leading to changes in the structure and function of cells (Metnitz et al., 1999; Zhang et al., 2000; Genestra, 2007). Antioxidant therapy has been useful in the prevention and the treatment of ALI/ARDS in some animal models (Brower et al., 2001; Bhatia and Moochhala, 2004; Wheeler and Bernard, 2007). *N*-Acetylcysteine (NAC) is a thiol compound which by providing sulfhydryl groups, can act both as a precursor of reduced glutathione and as a direct ROS scavenger, consequently regulating the redox status in the cells. In this way, it can interfere with several signaling pathways that play a role in regulating apoptosis, angiogenesis, cell growth and arrest, redox regulated gene expression, and inflammatory response (Atkinson, 2002). In clinical studies examining the role of *N*-acetylcysteine in ALI/ARDS and sepsis, there were encouraging trends in important clinical outcomes in patients who received the antioxidant but it did not produce effects that were significantly different from the placebo (Jepsen et al., 1992; Spies et al., 1994). Among several factors, the basis for these observations stem from the fact that the half-life of exogenously administered antioxidants is short and, for some, their high molecular weights do not allow them to cross cell membrane barriers (Muzykantov, 2001; Christofidou-Solomidou and Muzykantov, 2006). Encapsulation of antioxidants within liposomes greatly increases their intracellular delivery to specific target cells and enhances their protective effects against intracellular-oxidant mediated damage (Muzykantov, 2001; Suntres, 2002; Christofidou-Solomidou and Muzykantov, 2006).

Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules can be encapsulated in the aqueous spaces and lipophilic molecules can be incorporated into the lipid bilayers. Liposomes have successfully been used for the selective delivery of antioxidants and other therapeutic drugs to different tissues in sufficient concentrations to be effective in ameliorating tissue injuries (Suntres, 2002; Ratnam et al., 2006). The liposomal system is highly desirable for drug delivery because of: the relative ease in incorporating hydrophilic and lipophilic therapeutic agents in liposomes; the possibility of directly delivering liposomes to an accessible body site, such as the lung; and, the relative non-immunogenicity and low toxicity of liposomes (Suntres, 2002; Ratnam et al., 2006). In this study, the antioxidant effects *N*-acetylcysteine and liposome-entrapped *N*-acetylcysteine (L-NAC) were evaluated and compared in experimental animals challenged with lipopolysaccharide (LPS).

2. Materials and methods

2.1. Chemical products

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Northern Lipids (Vancouver, Canada). Myeloperoxidase (MPO), thromboxane B_2 and leukotriene B_4-enzyme-linked immunosorbent assay kits were purchased from R&D Systems (Minneapolis, MN, USA). *N*-Acetylcysteine and all other chemicals were purchased from Sigma–Aldrich Co. (Mississauga, Ontario, Canada).

2.2. Preparation of N-acetylcysteine liposomes

NAC liposomes were prepared from a mixture of DPPC and NAC in a 7:3 molar ratio by using a dehydration–rehydration method. Briefly, the lipid was dissolved in chloroform in a 50-mL round-bottomed flask and dried at 45 °C with a rotary evaporator (Buchi Rotavapor R 205). The lipid film was dried with nitrogen to eliminate traces of chloroform and hydrated with a 2 mL sucrose–water solution (1:1, w/w DPPC:sucrose) and subsequently sonicated (Model 500 Dismembrator, Fisher Scientific) for 5 min (cycles of 40 s on and 20 s off). After formation of multilamellar vesicles, NAC was added to the solution and freeze-dried overnight. Upon rehydration, free NAC was separated by centrifugation at 24,000 × *g* at 4 °C, for 30 min, a step performed twice. Liposomal vesicle size was determined with a Submicron Particle Sizer (Nicomp Model 270) after rehydration and was found to have an average mean diameter of 200 nm. The encapsulation efficiency of NAC by DPPC-liposomes was measured as 13 ± 4%.

2.3. Animals

Male Sprague–Dawley rats (approximate body weight of 225 g) were purchased from Charles River Canada, Inc. (St. Constant, Quebec). All animals were kept in autoclaved cages with free access to Purina laboratory chow and tap water. The animals were exposed to alternate cycles of 12-h light and darkness at room temperature (22–23 °C). The animals were cared for in accordance with guidelines provided by the *Canadian Council on Animal Care*.

2.4. Treatment of animals

Animals were anesthetized with ketamine and xylazine (15:1 ratio) under a protocol approved by the veterinarian. Animals were pretreated intravenously via the tail vein with NAC (25 mg/kg body weight; 0.5 mL), liposomal-NAC (25 mg/kg body weight; 1.3 mL), sterile saline (SAL), or empty liposomes (750 mg lipid/kg body weight). Four hours later, a single dose of LPS (1 mg/kg body weight *E. coli* 0111:B4) was injected intravenously to the animals via the tail vein. LPS was dissolved in saline shortly before use. Control animals received equal volumes of sterile saline.

2.5. Experimental design

To examine whether NAC administered as a liposomal formulation was more effective than its conventional form in attenuating the LPS-induced lung injuries, rats pretreated with L-NAC, NAC, empty liposomes or SAL were challenged with a single dose of LPS and killed 20 h later. The prophylactic effect of the antioxidant formulations against LPS-induced lung injury was assessed by measuring the wet weights of lungs as an indicator of edema and the activity of angiotensin-converting enzyme (ACE) in lung homogenates as an indicator of pulmonary endothelial cell integrity. The infiltration and activation of neutrophils in lungs were assessed by measuring the activity of myeloperoxidase (MPO) and concentration of chloramines in lung homogenates. Plasma levels of TNFα, and levels of thromboxane B_2 and leukotriene B_4 in bronchoalveolar lavage fluids were measured as indicators of the extent of the pro-inflammatory response. The extent of oxidative stress was assessed by measuring the levels of lipid peroxidation products (MDA and 4-HNE) and non-protein thiols (NPSH) in lung homogenates (Suntres and Shek, 2000; Suntres et al., 2002; Suntres, 2003).

2.6. Tissue preparation

Blood samples were collected from animals by cardiac puncture in vacutainer tubes under anesthesia. The collected blood was centrifuged immediately (1300 × *g* for 15 min at 4 °C) and the isolated plasma was stored at −80 °C and used within 48 h for the determination of TNFα. Lungs from animals were removed 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, organs were quickly weighed and finely minced. Approximately 1 g of organ sample was homogenized with a Brinkmann

Polytron (Brinkmann Instruments) in a sufficient volume of ice-cold 50 mM potassium phosphate buffer, pH 7.4, to produce a 20% homogenate. For the measurement of lipid peroxidation products, homogenates were prepared as described previously except the homogenizing medium contained 3 mM EDTA.

2.7. Isolation of bronchoalveolar lavage fluid (BALF)

BALF from both lungs was performed via an intratracheal cannula with a total of 10 mL of saline in 4 mM aliquots. Recovered lavage fluid was centrifuged at 400 × *g* for 10 min, and the supernatant was collected and stored at −80 °C for use in measuring thromboxane B_2, leukotriene B_4, and *N*-acetylcysteine levels.

2.8. Enzyme measurements

The activity of MPO in sonicated whole lung homogenates was estimated by using a specific enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). Measurement of TNFα concentrations in plasma, thromboxane B_2 and leukotriene B_4 in bronchoalveolar lavages and ACE activities in lung homogenates was carried out by using a specific enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions.

2.9. Determination of lung levels of lipid peroxidation and non-protein thiols (NPSH)

Lung homogenates from treated and control animals were assayed for the presence of products of lipid peroxidation (MDA and 4-HNE) by using a colorimetric assay (Bioxytech LPO-586, Oxis International, Forest City, CA). Non-protein sulfhydryls in pulmonary homogenates were determined as described by Suntres and Lui (Suntres and Lui, 2006). Briefly, the tissue was homogenized in 20% (w/v) trichloroacetic acid and centrifuged at 9000 × *g* for 20 min. An aliquot of the supernatant fraction in 0.3 M phosphate buffer was treated with 5,5-dithiobis-[2-nitrobenzoic acid] and the absorbance at 412 nm was measured.

2.10. Measurement of NAC by ultra-performance liquid chromatography (UPLC)

UPLC separation was performed using a Waters Acquity system equipped with a binary solvent manager, an automated sample manager, and a photodiode array detector. Samples were filtered through a 0.2 μm filter and injected onto an Acquity UPLC HSS T3 analytical column (2.1 mm × 150 mm, 1.8 μm particles), with a Vanguard 2.1 mm I.D. × 5 mm length guard column, at 30 °C. The mobile phase consisted of 23 mM ammonium formate (pH 3), at a flow rate of 0.250 mL/min. NAC was measured at a wavelength of 200.3 nm.

2.11. Statistical analysis

Data from control and experimental animals pretreated with saline, empty liposomes, NAC or L-NAC and challenged with LPS were evaluated by one-way analysis of variance (ANOVA). If the *F* values were significant, the Newman–Keuls *t*-test was used to compare all groups. The level of significance was accepted at $p < 0.05$.

3. Results

3.1. Lung weight and changes in pulmonary ACE activity

The lung weights of animals pretreated with saline, conventional NAC, empty liposomes, or L-NAC and challenged with LPS are shown in Fig. 1. Challenge of animals with LPS resulted in significant increases in wet lung weights (44% of control value) suggestive of pulmonary edema and infiltration. Pretreatment of animals with conventional NAC was not effective in reducing the LPS-induced edema to a significant degree. On the other hand, pretreatment of animals with L-NAC was effective in preventing LPS-induced increases in lung weights (15% of control value).

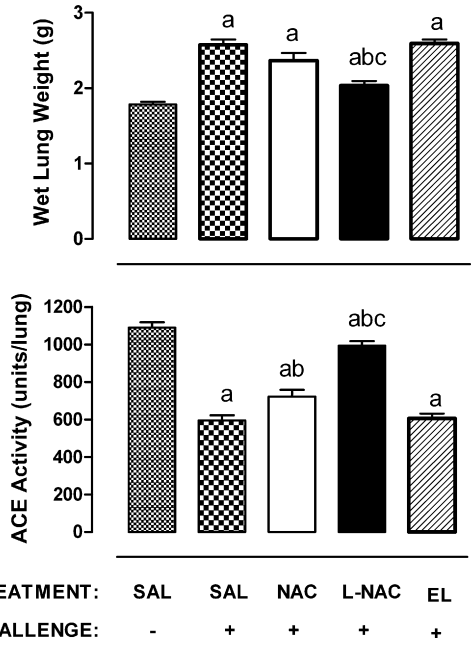

Fig. 1. The effect of NAC-pretreatment on LPS-induced changes in lung weights (upper panel) and angiotensin-converting enzyme (ACE) activities. NAC (25 mg/kg, iv) was administered to rats either in its conventional or liposomal form and 4 h later animals were challenged with LPS (1 mg/kg, iv); animals were sacrificed 20 h later. Each data point represents the mean ± S.E.M. of five animals: (a) denotes a significant difference ($p < 0.05$) compared with the mean value of the saline (SAL) treated group; (b) denotes a significant difference ($p < 0.05$) compared with the mean value of the group of animals pretreated with saline and challenged with LPS; (c) denotes a significant difference ($p < 0.05$) compared with the mean value of the group of animals pretreated with free NAC and challenged with LPS.

The ACE activity in lungs of animals challenged with LPS was significantly decreased suggestive of pulmonary endothelial cell injury (Lazo et al., 1986). Pretreatment of animals with the antioxidant formulations was effective in protecting against LPS-induced decreases in ACE activities with the protective effect of L-NAC being far superior to that seen following pretreatment with conventional NAC (Fig. 1).

3.2. Changes in tumour necrosis factor-alpha levels (TNFα)

The administration of LPS to saline pretreated animals resulted in significant increases (4.12-fold) in plasma TNFα levels (Fig. 2). Pretreatment of animals with either NAC or empty liposomes did not alter the LPS-induced increases of TNFα levels in plasma. Pretreatment with L-NAC, however, significantly reduced the LPS-induced increases in plasma TNFα levels (Fig. 2).

3.3. Changes in pulmonary levels of myeloperoxidase and chloramine

Infiltration and activation of phagocytes in the lungs of LPS-challenged animals were assessed by measuring the activities of myeloperoxidase (MPO) and chloramine concentrations (long-lived oxidants produced by neutrophils) (Witko-Sarsat et al., 1995). As shown in Fig. 2, MPO activities in LPS-challenged animals were

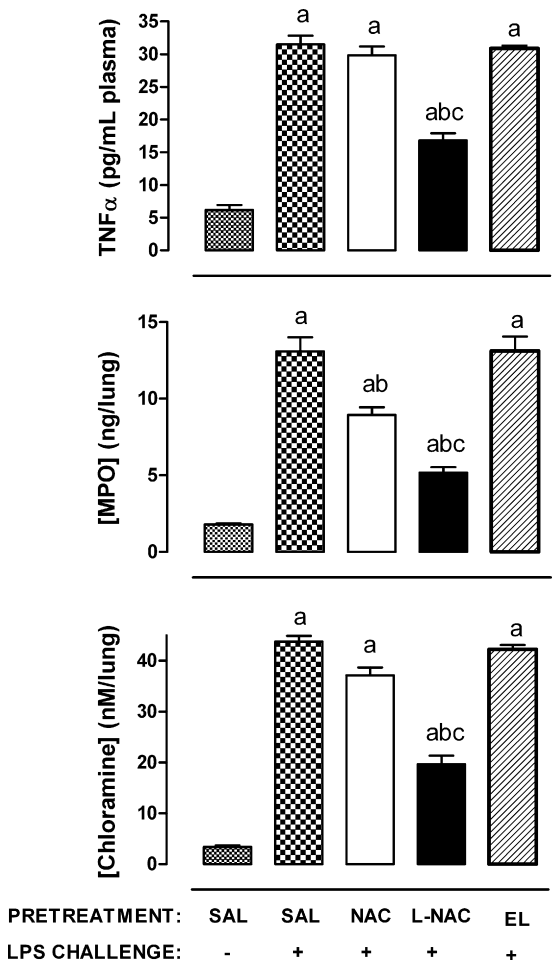

Fig. 2. The effect of NAC-pretreatment on LPS-induced changes in plasma TNF-α levels (upper panel) and pulmonary myeloperoxidase (MPO) activities (middle panel) and chloramine contents (lower panel). NAC (25 mg/kg, iv) was administered to rats either in its conventional or liposomal form and 4 h later animals were challenged with LPS (1 mg/kg, iv); animals were sacrificed 20 h later. Each data point represents the mean ± S.E.M. of five animals: (a) denotes a significant difference ($p < 0.05$) compared with the mean value of the saline (SAL) treated group; (b) denotes a significant difference ($p < 0.05$) compared with the mean value of the group of animals pretreated with saline and challenged with LPS; (c) denotes a significant difference ($p < 0.05$) compared with the mean value of the group of animals pretreated with free NAC and challenged with LPS.

significantly elevated in the lungs (6.3-fold) suggestive of neutrophil infiltration. Also, LPS challenge of saline-pretreated rats resulted in significant increases in pulmonary chloramine concentrations suggestive of phagocyte activation. Pretreatment of animals with NAC or empty liposomes failed to alter the LPS-induced increases in MPO and chloramine levels. However, the levels of these markers were significantly lower in the lungs of LPS-challenged animals pretreated with L-NAC.

3.4. Changes in thromboxane B_2, leukotriene B_4, and NAC levels in bronchoalveolar lavage fluids (BALF)

Prostaglandins and leukotrienes are potent eicosanoid lipid mediators derived from phospholipase-released arachidonic acid and established pro-inflammatory mediators that are involved in numerous homeostatic biological functions and inflammation (Funk, 2001). LPS administration to saline-pretreated animals resulted in significant increases in pulmonary levels of thromboxane B_2 and leukotriene B_4 (Fig. 3C and D). Pretreatment of animals with L-NAC significantly reduced the pulmonary levels of the pro-inflammatory mediators generated following exposure to LPS while pretreatment of animals with either NAC or empty liposomes failed to produce a protective effect (Fig. 3C and D).

Since oxidant stress occurs within the alveolar compartment, *N*-acetylcysteine (NAC) levels were measured in the bronchoalveolar lavage fluid (BALF) of animals with acute lung injury. As shown in Table 1, the levels of NAC in the BALF of L-NAC pretreated animals were significantly higher than those observed following NAC pretreatment.

3.5. Changes in pulmonary levels of lipid peroxidation products and non-protein thiols (NPSH)

Since lipid peroxidation of membrane lipids has been implicated as a possible mechanism of acute oxidative stress-induced lethal injury, the products of lipid peroxidation in tissues have been used as a measure of oxidative stress (Rahman, 2006). In the present study, animals challenged with LPS produced a significant increase in the pulmonary levels of lipid peroxidation products (6.1-fold), as measured by the formation of MDA and 4-HNE (Fig. 3A). Although pretreatment of animals with NAC or empty liposomes did not alter the LPS-induced increases in pulmonary lipid peroxidation levels, administration of L-NAC prior to LPS challenge significantly reduced the extent of lipid peroxidation in liver tissues.

A decrease in lung tissue NPSH, the major thiol component being reduced glutathione (GSH) which accounts for over 90% of the intracellular NPSH, is considered to represent an index of oxidative stress (Patsoukis and Georgiou, 2004; Glantzounis et al., 2006). In the present study, the levels of NPSH in lung homogenates obtained from animals challenged with LPS were significantly reduced (Fig. 3B). The LPS-induced decreases in pulmonary NPSH levels were not significantly affected by prior treatment with empty liposomes or NAC. On the other hand, pretreatment of animals with L-NAC ameliorated the LPS-induced decreases in pulmonary NPSH levels (Fig. 3B).

4. Discussion

The results of the present study showed that the production of ROS is important in the pathogenesis of acute lung injury observed following LPS exposure. This is consistent with our finding where LPS administration resulted in increases in the levels of lipid peroxidation assessed by measuring the products 4-hydroxy-2-nonenal and MDA. Lipid peroxidation is recognized as an indicator of oxidative stress and a potential mechanism of tissue damage associated with several inflammatory disorders including ARDS (Zhang et al., 2000; Bhatia and Moochhala, 2004; Rahman, 2006). Experimental evidence have shown that, in comparison with normal healthy control participants, patients with ARDS have higher circulating levels of 4-hydroxy-2-nonenal, a marker of ROS-mediated oxidation of the essential fatty acid linoleic acid (Quinlan et al., 1994). Similarly, challenge of experimental animals with LPS resulted in increases in tissue lipid peroxidation levels which were closely associated with the production of ROS (Thorn, 2001; Lang et al., 2002; Tasaka et al., 2008). The source of ROS that cause lipid peroxidation can be generated mostly from macrophages and neutrophils and to a lesser extent from pulmonary epithelial and endothelial cells (Thorn, 2001; Lang et al., 2002; Tasaka et al., 2008).

An extensive body of evidence shows that ARDS can be prevented or ameliorated in numerous different animal models by timely pharmacologic intervention with agents that inhibit the synthesis of ROS and/or RNS, or scavenge these reactive molecules once they have been formed (Metnitz et al., 1999; Zhang et al.,

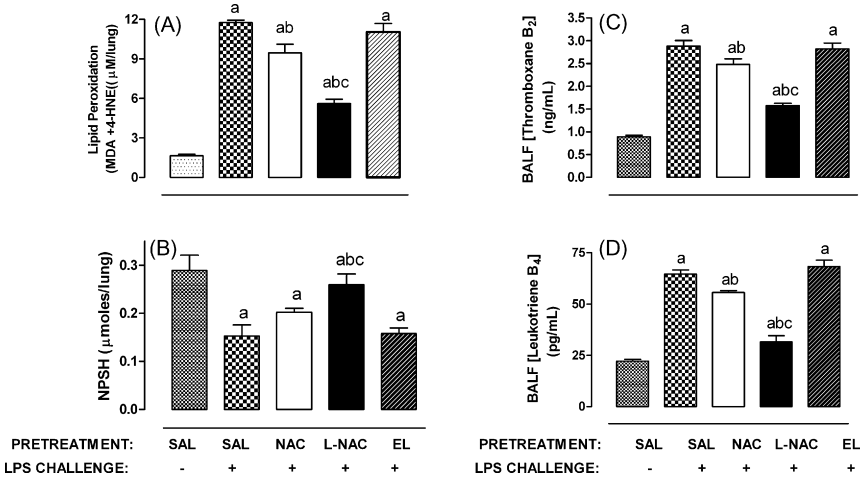

Fig. 3. The effect of NAC-pretreatment on LPS-induced changes in lipid peroxidation (A) and non-protein thiols (NPSH) (B) in lung homogenates and thromboxane B_2 (C) and leukotriene B_4 (D) levels in broncoalveolar lavage fluid. NAC (25 mg/kg, iv) was administered to rats either in its conventional or liposomal form and 4 h later animals were challenged with LPS (1 mg/kg, iv); animals were sacrificed 20 h later. Each data point represents the mean ± S.E.M. of five animals: (a) denotes a significant difference ($p < 0.05$) compared with the mean value of the saline (SAL) treated group; (b) denotes a significant difference ($p < 0.05$) compared with the mean value of the group of animals pretreated with saline and challenged with LPS; (c) denotes a significant difference ($p < 0.05$) compared with the mean value of the group of animals pretreated with free NAC and challenged with LPS.

2000; Brower et al., 2001; Atkinson, 2002; Bhatia and Moochhala, 2004; Genestra, 2007; Wheeler and Bernard, 2007). To neutralize free radicals and counteract their detrimental effects produced during inflammatory processes, cells express a number of endogenous antioxidants such as glutathione, superoxide dismutase, catalase, and glutathione peroxidase (Muzykantov, 2001; Suntres, 2002; Christofidou-Solomidou and Muzykantov, 2006; Ratnam et al., 2006). However, these antioxidants are rapidly overwhelmed during an acute inflammatory response, consistent with the results presented in this study where LPS administration resulted in decreases in the pulmonary levels of non-protein thiols (Fig. 3). Other studies have also shown that LPS administration has resulted in reduction of cellular antioxidants including glutathione, alpha-tocopherol, vitamin C, and beta-carotene (Cadenas and Cadenas, 2002; Macdonald et al., 2003; Victor et al., 2004).

Administration of the antioxidant *N*-acetylcysteine, known to inhibit the toxic effects of ROS, attenuated acute lung injury induced following LPS administration. The protective effect of *N*-acetylcysteine was greater in animals pretreated with the liposomal NAC formulation, a treatment effect attributed to the ability of liposomes to deliver higher levels of the antioxidant to the lung. This is consistent with our finding that the levels of non-protein thiols in lung homogenates of animals pretreated with liposomal NAC were higher than those observed following administration of conventional NAC (Fig. 3). Furthermore, measurement of NAC in lung bronchoalveolar lavage fluid showed that the levels of NAC in the group of animals pretreated with L-NAC were significantly higher than those pretreated with NAC (Table 1). It has been reported that following oral or intravenous administration of NAC, the levels of the antioxidant or its metabolites in lung tissues or bronchoalveolar fluid are not significantly increased (Sadowska et al., 2007). In our study, it appears that intravenous administration of L-NAC prolonged the half-life and hence the bioavailability of NAC for distribution to the lung. In a separate study examining the safety and pharmacokinetics of L-NAC in control rats, it has been demonstrated that the half-life of intravenously administered L-NAC is significantly increased by fourfold from 6 to 30 min (unpublished observation). Similarly, results presented by other investigators have shown that intravenous administration of drugs as liposomal formulations prolong their circulation time in blood and increase their distribution to major organs, including the lung (Suntres, 2002; Ratnam et al., 2006).

Table 1
NAC concentrations in the bronchoalveolar fluid (BALF) from lungs of animals pretreated with conventional NAC or L-NAC and challenged with LPS as described in Section 2

Treatment Group	NAC (μM)
NAC + LPS	0.10 ± 0.05
L-NAC + LPS	0.68 ± 0.02[a]

[a] Denotes a significant difference ($p < 0.001$) compared with the mean value of the group of animals pretreated with conventional NAC and challenged with LPS.

Depletion of non-protein thiols, the most important being GSH, during inflammation renders cells susceptible to the amplification of inflammatory responses. Redox-sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (c-Fos/c-Jun, AP-1) are known to play a key role in pro-inflammatory processes such as the transcription of cytokine genes and in upregulating protective antioxidant genes (Liu et al., 1999; Cadenas and Cadenas, 2002; Macdonald et al., 2003). In view of the fact that antioxidants regulate gene activation mediated through an inhibitory effect on NF-κB (Cadenas and Cadenas, 2002; Macdonald et al., 2003; Liu et al., 1999; Thakur et al., 2006), we postulated that administration of L-NAC might lessen lung injury by preventing the induction of genes implicated in the development and perpetuation of the inflammatory response, presumably by down-regulating NF-κB. Specifically, we measured the induction of the pro-inflammatory cytokine TNFα since it has been implicated in the development of acute lung injury, and induction of their respective genes has been shown to be sensitive to antioxidants *in vitro* (Marui et al., 1993; Liu et al., 1999). Our data demonstrated that L-NAC was more effective than conventional NAC in decreasing the levels of TNFα *in vivo*, most likely by maintaining the cellular thiol status close to normal. TNFα has been recognized as the primary and central cytokine in mediating an early inflammatory response, characterized mainly by neutrophil infiltration and activation (Sury et al., 2006; Thakur et al., 2006). Our data are consistent with the fact that the decreases in plasma TNFα levels are most likely responsible for the reduction in neutrophil infiltration and activation, a treatment effect supported by our findings in that L-NAC suppressed the LPS-induced increases in MPO activity (an

enzyme primarily localized in neutrophils Bradley et al., 1982). As a consequence to a decrease in the accumulation and activation of neutrophils in the lung is a reduction of the availability of toxic metabolites released from phagocytic cells, such as in the case of hypochlorous acid, an oxidant formed by neutrophilic myeloperoxidase, which reacts with free amino acids forming chloramines (Witko-Sarsat et al., 1995).

In addition to the decrease in the production of TNFα produced following LPS administration, L-NAC exerted its anti-inflammatory effects by preventing the increases of other pro-inflammatory mediators, such as thromboxane B_2, a stable metabolite of thromboxane A_2 and leukotriene B_4. In this study, LPS administration, resulted in an increase in wet lung weight, a treatment effect attributed to increases in lung permeability, protein leakage and PMN infiltration consistent with marked increases in thromboxanes and leukotrienes detected in lung BALF. Thromboxane A_2 may increase lung permeability whereas leukotriene B_4 is a potent neutrophil chemoattractant (Funk, 2001). The mechanism(s) by which NAC prevented the increases of the pro-inflammatory mediators are not understood from the results of this study but NAC might exert its anti-inflammatory effect by decreasing cell membrane damage and the associated release of phospholipids. Free radicals and lipid peroxidation products are known to generate reversible changes in cellular unsaturated fatty acids which are responsible for activation of phospholipases and subsequent release of substrates for the lipoxygenase pathway (Starkopf et al., 1998). Our data showed the L-NAC was able to decrease the levels of lipid peroxidation.

In conclusion, pretreatment with an intravenous bolus of NAC at a dose of 25 mg/kg body weight as a liposomal formulation, attenuated the LPS-induced lung injuries as evaluated by the changes in lung weight, biochemical, and pro-inflammatory parameters. Previous studies have shown that doses greater than 200 mg/kg NAC per day are required in order for conventional NAC to exert its antioxidant and anti-inflammatory effects in experimental animal models or patients with associated lung injuries (Sadowska et al., 2007). Also, administration of plain liposomes, without any entrapped NAC, failed to attenuate the LPS-induced lung injuries suggesting that the lipid components of the liposomal vehicle do not possess any antioxidant or anti-inflammatory properties. It appears that L-NAC ameliorated the LPS-induced lung injuries by lessening the effects of ROS and inflammation, a treatment effect attributed to the higher antioxidant delivery and retention of NAC in the lung.

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