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Authors

Suresh, Madathilparambil V
Yu, Bi
Lakshminrusimha, Satyan
et al.

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The protective role of MnTBAP in Oxidant-mediated injury and inflammation following Lung Contusion

Madathilparambil V Suresh[1], **Bi Yu**[1], **Satyan Lakshminrusimha**[3], **D Machado-Aranda**[1], **Nicholas Talarico**[1], **Lixia Zeng**[2], **Bruce A. Davidson**[4], **Subramaniam Pennathur**[2], and **Krishnan Raghavendran**[1]

[1]Department of Surgery, University of Michigan, Ann Arbor MI

[2]Department of Internal Medicine, Division of Nephrology, University of Michigan

[3]Departments of Pediatrics, University at Buffalo, Buffalo NY

[4]Department of Anesthesiology, University at Buffalo, Buffalo, NY

Abstract

Background—Lung contusion (LC) is a unique direct and focal insult that is considered a major risk factor for initiation of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). We have recently shown that consumption of Nitric oxide (NO)(due to excess superoxide) resulting in peroxynitrite formation leads to diminished vascular reactivity after LC. Here, we set to determine if superoxide scavenger Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) plays a protective role in alleviating acute inflammatory response and injury in LC.

Methods—Non-lethal closed-chest bilateral lung contusion was induced in a rodent model. Administration of superoxide dismutase (SOD) mimetic-MnTBAP, concurrently with LC in rats was performed and bronchoalveolar lavage (BAL) and lung samples were analyzed for degree of injury and inflammation at 5 and 24 h following the insult. The extent of injury was assessed by the measurement of cells and albumin with cytokine levels in the BAL and lungs. Lung samples were subjected to H&E and superoxide staining with dihydro-ethidium (DHE). Protein-bound dityrosine and nitrotyrosine levels were quantified in lung tissue by tandem mass spectrometry.

Results—The degree of lung injury after LC as determined by BAL albumin levels were significantly reduced in the MnTBAP administered rats at all the time points, when compared to the corresponding controls. The release of pro-inflammatory cytokines and BAL neutrophils were significantly lower in the MnTBAP administered rats after LC. Pathological examination revealed that administration of MnTBAP reduced tissue damage with decreased necrosis and neutrophil-rich exudate at the 24 h time point. Staining for superoxide anions showed significantly higher intensity in the lung samples from LC group compared to LC+ MnTBAP. Liquid chromatography/

Corresponding author: Krishnan Raghavendran, M.D., Department of Surgery, (Division of Acute Care Surgery), University of Michigan, 1500 E. Medical Center Drive, 1 C421-UH, Ann Arbor, MI 48109. kraghave@umich.edu.

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tandem mass spectrometry [HPLC/MS/MS] revealed that MnTBAP treatment significantly attenuated dityrosine and nitrotyrosine levels consistent with reduced oxidant injury.

Conclusion—SOD mimetic-MnTBAP reduced permeability and oxidative injury in LC and may have a therapeutic role in diminishing inflammation in LC.

Introduction

Blunt chest trauma is involved in nearly one-third of acute trauma admissions to the hospital, and lung contusion (LC) is an independent risk factor for the development of acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and ventilator-associated pneumonia (VAP) [1,2]. The lung is also the second commonest organ involved in blast trauma-induced LC, which often has a peri-hilar distribution and carries a high risk of mortality [3]. When LC injury leads to hypoxemia severe enough to meet the definition of ALI/ARDS, the prognostic and economic impacts are significant. These clinical syndromes continue to have very substantial overall mortality and morbidity despite significant advances in cardio-respiratory intensive care over the past several decades [4]. In a 2004 study of trauma patients, the incremental hospital cost per patient with ALI or ARDS ($36,713 or $59,633, respectively) was much higher than for patients without ALI/ARDS ($24,715) [5].

Pulmonary contusion is characterized by an injury to the lung parenchyma, causing disruption of alveolar walls. This leads toedema and collection of blood in the alveolar spaces with loss of normal lung structure and function. As a result, there is poor gas exchange, increased pulmonary vascular resistance, and decreased lung compliance. LC is also an independent risk factor for the development of acute respiratory failure manifesting as clinical acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and ventilator-associated pneumonia. Previously, we have shown that ALI developed from LC is neutrophil dependent[6]. We documented the recruitment and activation of neutrophils and lung tissue macrophages, as well as the production of multiple cytokines and chemokines in LC[7]. We and others have reported on the time course and pathophysiology of isolated LC induced by closed-chest blunt trauma in rodent models (rats and mice)[6–12].

Nitric oxide (NO) is a ubiquitous molecule needed for normal physiologic functions and is generated from L-arginine through an oxidation reaction that is catalyzed by NO synthase (NOS). The inducible form of NOS (iNOS, NOS2) can be expressed in many cell types, and it also exhibits immunosuppressive properties that may play a role in the down-regulation of immune responses. NO is a weak free radical, but in combination with other ROS, such as superoxide (O^{2-}), it can result in the formation of peroxynitrite ($ONOO^{-}$), a toxic and reactive product capable of mediating cytotoxic processes[13–15]. One of the key reactions of $ONOO^{-}$ in biological systems is the reaction with carbon dioxide, which leads to the formation of carbonate ($^{\bullet}CO_3^{-}$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals. Nitrogen dioxide can undergo diffusion controlled radical-radical termination reactions with biomolecules, resulting in nitrated species such as nitrotyrosine or peroxynitrite[16,17].

The local bioavailability of NO in tissues is determined by the local concentration of superoxide anions and by the activity of antioxidant enzymes such as SOD and catalase, which scavenge superoxide and prevent formation of peroxynitrite. Our recent data suggest that consumption of NO due to excess superoxide resulting in peroxynitrite formation leads to abnormal vascular reactivity (enhanced constriction to norepinephrine and impaired relaxation to NO donors) after LC[18]. Additionally, we concluded that compensatory increases in cytosolic SOD improve arterial reactivity and hypoxia at 24 h afterLC[18].

In the current study, we investigate the biological role of SOD by using MnTBAP [Mn (III) tetrakis (4-benzoic acid) porphyrin chloride], a low molecular weight synthetic non-peptide

mimetic of SOD and studied its effects after LC in rats. Unlike recombinant SOD, which cannot cross biological membranes, MnTBAP is non-immunogenic and crosses the plasma membrane to neutralize superoxide in both extracellular and intracellular compartments. It overcomes the extremely short half-life of scavenging activity of natural SOD and may have a wider clinical application[19,20]. These manganic porphyrins catalyze the dismutation of superoxide radical (with rate constants of ~ 10^7 M/s) and possess the ability to protect a SOD-null strain of *E. coli* against dissolved oxygen. MnTBAP is reduced enzymatically at the expense of NADPH and non-enzymatically by GSH and maintained in a reduced state. It is thought that this compound may act as a NADPH/GSH:O_2/-oxidoreductase rather than just an SOD mimic[21]

MnTBAP may also inhibit the oxidation of dihydrorhodamine-123 elicited by authentic peroxynitrite[16,21–23]. This effect is important in the context of our studies that demonstrated that tyrosine oxidation markers (nitrotyrosine and dityrosine) were markedly increased after LC, consistent with increased oxidant stress mediated by peroxynitrite. In the present study, we hypothesized that MnTBAP, when administered with LC, will decrease permeability injury and inflammation after LC. We report that MnTBAP treatment attenuated injury and acute inflammation after LC in a rodent model, raising the possibility that this mechanism could be exploited as a therapeutic tool to prevent ALI after LC.

Materials and Methods

a. Animals

Adult, male, Long-Evans rats (280–300 g body wt., Harlan Sprague-Dawley, Indianapolis, IN) were utilized. All procedures performed were approved by the Institutional Animal Care and Use Committee at the University of Michigan, and complied with State, Federal, and National Institutes of Health regulations.

b. Induction of bilateral closed-chest LC

LC was induced in halothane-anesthetized rats by dropping a hollow, aluminum, cylindrical weight (300 g) from a height of 80 cm onto the chest as described before[6]. The key part of the instrument is the presence of a protective plastic shield that protects the mediastinal structures and selectively injures the lungs bilaterally[6]. This protocol results in impact energy of 2.2 J and is associated with low mortality (2/38 rats). Twelve rats that did not undergo contusion were used as controls. We have been using a similar protocol for about 7 years and typically, severely injured rats do not wake up from anesthesia after the induction of LC[6,24,25]. Animals that demonstrate spontaneous respirations and wake up tend to live longer periods of time (observed as long as 7 days in our laboratory) without oxygen supplementation.

c. Administration of anesthetic, analgesic and resuscitation

The animal was anesthetized by intra-peritoneal (i.p) injection of ketamine (80–120 mg/kg body wt.) and xylazine (5–10 mg/kg body wt.). We did not use any systemic analgesics due to their effects on the immune/inflammatory system. In the event of severe respiratory distress beyond 48 h, these animals were humanely euthanized.

d. Administration of cell permeable MnTBAP

MnTBAP was administered intra-peritoneally at the dose of 10 mg/kg body weight during the same anesthetic period used for induction of LC. Animals were divided randomly into four groups. Each experiment was repeated at least three times with 3–5 animals per group to get n=9–12. The two time points (5 hours and 24 hours) reflect different

pathophysiological aspects of LC[25]. The 5-hour time point represents a degree of maximal hypoxemia and structurally is characterized by alveolar disruption and permeability injury as a result of mechanical disruption.

The 24 h time point represents partial reversal of hypoxemia. Additionally, it represents a time point with peak inflammation and therefore permeability injury[25].

Group 1:	Control – no contusion, saline i.p. injection.
Group 2:	LC – contusion alone harvested at 5 and 24 h time points.
Group 3:	LC + concurrent MnTBAP harvested at 5 and 24 h time points.
Group 4:	MnTBAP alone harvested at 5 and 24 h time points.

e. Broncho-alveolar lavage

In order to determine albumin and cytokine levels in the lungs after LC, bronchoalveolar lavage (BAL) was obtained at various time points. After anesthesia, a midline incision was made through the sternum and the lung vasculature was flushed by injecting 100 mL of phosphate-buffered saline (PBS) solution into the beating right ventricle. BAL was performed using 2 × 5 m Laliquots of PBS through the tracheal cannula using a 10 ml syringe. Recovered BAL fluid was centrifuged and the supernatant was frozen for later albumin and cytokine analyses [25].

f. Albumin concentrations in BAL

Albumin concentrations in the BAL fluid were measured by ELISA using a polyclonal rabbit anti-rat albumin antibody; a HRP-labeled goat anti-rabbit IgG (BD Biosciences Pharmingen, San Diego, CA), and rat albumin (Sigma, St. Louis, MO) was used as a standard.

g. Determination of cytokine levels in BAL

Concentrations of interleukin [IL] 1β, IL-6, IL-10, Cytokine-induced neutrophil chemoattractant 1[CINC-1] and CINC-3 in BAL and lung samples were determined using ELISA. Antibody pairs (one capture antibody and one biotinylated-reporter antibody) and recombinant cytokines for these assays were obtained from R&D Systems (Minneapolis, MN) and were measured based on the manufacturer's instructions.

h. Cell differential count by Cytospin

For cytospin preparations, BAL cells were centrifuged at 600 × g for 5 min using a cytospin II (Shandon Scientific, Pittsburgh, PA), stained with Diff-Quik (Dade Behring Inc., Newark, DE) and analyzed by examining under a light microscope at 200 × magnification.

i. Quantitative analysis of tyrosine oxidation products by high performance liquidchromatography Tandem mass spectrometry (HPLC/MS/MS)

Plasma, protein-bound oxidized amino acids dityrosine and nitrotyrosine were measured by HPLC/MS/MS using a triple quadruple mass spectrometer as described previously[26]. Briefly, the tissue was homogenized, and then the protein pellet was isolated. Isotopically labeled internal standards were added, and samples were hydrolyzed with 4N methane sulfonic acid at 110°C for 24 hours under argon. Quantification of oxidized amino acids were performed using isotope dilution electrospray ionization MS as described previously[26].

j. Histopathology

Lung specimens harvested at time of death were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. Slides were evaluated by an experienced, blinded pathologist and graded for the presence of interstitial neutrophilic infiltrate, intra-alveolar hemorrhage, and pulmonary septal edema, as described previously[27].

k. Staining for DHE

Lung sections were prepared and stained as described previously[28]. The right middle lobe of the rat lung was removed, and OCT compound (Tissue–Tek, Torrance CA) was pushed gently into the deflated lobe and allowed to solidify on ice for 15–20 min. Blocks were prepared and cut into 30 μm sections that were mounted onto charged slides for staining and stored at −80°C. Frozen lung sections were fixed subsequently with acetone and exposed to 5 μM dihydroethidium (DHE; Molecular Probes/Invitrogen, Grand Island, NY) in PBS. Slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Ethidium-stained slides were observed by fluorescence microscopy with excitation at 518 nm and emission at 605 nm. Fluorescent images were captured using a Nikon Eclipse Ti Microscope with NIS elements AR imaging software (Nikon Inc., Melville, NY).

l. Statistical Analyses

All data are expressed as mean ± SEM, with ‘n’ representing the number of animals studied. Statistical analyses of inter-group comparisons were analyzed using the unpaired t-test with Welch’s correction (GraphPad Prism 5.01, GraphPad software, San Diego, CA).*$P < 0.05$ compared to corresponding treated animals unless specified[8].

Results

1. Effect of MnTBAP on permeability injury after LC

We assessed the BAL for albumin, an indicator of the extent of permeability injury. There was a marked increase in the BAL albumin level in the rats at 5 and 24 h after LC, and significantly decreased by MnTBAP at the 24 h time point (Fig. 1)

2. Cytokine and Cellular responses after MnTBAP administration

Lung injury during LC is characterized by an intense inflammatory response, which contributes to the physiological dysfunction in this condition; this process involves production of mediators such as chemokines and cytokines. In order to determine if MnTBAP administration has any role in the production of these mediators after LC, we measured the levels of pro-inflammatory interleukin [IL]-1β and IL-6. The levels of IL-1β in BAL and lungs were significantly decreased at 5 and 24 h rats administered with MnTBAP, when compared to control rats with LC (Fig. 2A and B). Similar results were obtained for IL-6 (Fig. 2C and D). We also measured the levels of anti-inflammatory (IL-10) cytokines as well ascytokine-induced neutrophil chemoattractants, CINC-1 and CINC-3. There was no significant difference in the levels of IL-10 between the two injury groups at 5 and 24 h after LC compared to the corresponding MnTBAP administered rats (Fig. 3A and B). The levels of cytokine-induced neutrophil chemoattractant (CINC-1) were significantly increased in rats at 5 and 24 h after LC, compared to the corresponding MnTBAP administered rats (Fig. 3C and D). CINC-3 levels also increased at 24 h prominently in contused rats without MnTBAP treatment (Fig. 3E and F). These results suggest that anti-oxidants such as superoxide dismutase play a role in down modulating the intensity of acute inflammation after LC.

Activation of tissue macrophages is observed in the lungs in response to a variety of inflammatory stimuli. We determined the levels of macrophages in BAL collected from rats at different time intervals using cytospin technique (Fig. 4A). The numbers of macrophages were significantly greater at 5 h post-LC and this effect was decreased significantly in the MnTBAP treated rats at the same time point. Similarly, we have shown previously that the acute inflammatory response in LC is neutrophil-dependent and was an important contributor of ongoing respiratory dysfunction[6,7]. Additional measurements of neutrophils in the BAL after LC in both MnTBAP treated and non-treated group were conducted using a cytospin. At 24 hours, there was a significant increase in neutrophil levels in the LC rats significantly attenuated by MnTBAP treatment (Fig. 4B). These results were further confirmed by cytospin (Fig. 4C).

3. Histology

Histological examination of rats 5 hours after injury (Fig. 5) showed there was a large area of intra-alveolar hemorrhage at the surface of the lung. After 24 hours of LC, in addition to areas of moderate intra-alveolar hemorrhages, there were areas of focal damage to the alveoli and consistent of hypertrophied epithelial cells and alveolar infiltrates of neutrophils. In the group of animals where MNTBAP was administered concurrently with LC, there was a large area of intra-alveolar hemorrhage at the surface of the lung as seen 5 h after LC, and the findings appear to be similar to the LC only group at the same time point. However in the group of animals with LC+MNTBAP at the 24 h time point, there was significantly less neutrophil-rich exudate (Fig. 5).

4. Superoxide anion staining in lung samples

Dihydroethidium (DHE) by virtue of its ability to freely permeate cell membranes is used extensively to monitor superoxide production. The in situ production of superoxide anion was measured in frozen lung sections after LC in both the MnTBAP treated and non-treated groups using the oxidative fluorescent dye dihydroethidium (DHE). The result show that pulmonary sections from LC after the administration of MnTBAP showed a decrease in DHE fluorescence intensity at 5 and 24 h compared to LC animals without any treatment (Fig. 6).

5. Decreased injury and inflammation after MnTBAP (SOD mimetic) is associated with diminished oxidant production after LC

Evidence suggests that products of tyrosine oxidation are sensitive markers of oxidative injury and may play an important role in many inflammation-related diseases[29,30]. To investigate the functional importance of oxidant production in LC after the use of MnTBAP concurrent with LC, the protein hydrolysates derived from the lung were subjected to high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS). As shown in Fig. 7A and B, the levels of nitrotyrosine and dityrosine after LC were significantly decreased. (Nitrotyrosine/Tyrosine concentrations in μM/mole; LC 5 h 0.62 ± 0.21, LC+ MnTBAP 5 h- 0.12 ± 0.04, LC 24 h- 0.47 ± 0.10 and LC + MnTBAP 24 h- 0.18 ± 0.03; Dityrosine/tyrosine concentrations; LC 24 h- 0.36 ± 0.05 and LC +MnTBAP 24 h-0.25 ± 0.02) in the MnTBAP administered group, at both 5 and 24 h time points compared to non-treated animals. As described previously[8,26], these markers are specific indicators of peroxynitrite formation (formed by NO and superoxide). These data indicate that scavenging superoxide decreases oxidative stress in LC, likely through diminished peroxynitrite formation.

Discussion

LC is a unique direct and focal insult that is considered a major risk factor for initiation of ALI/ARDS[1,2]. Moreover, the physical nature of the trauma with resulting tissue injury leading to inflammation makes it different from other etiological factors of ALI/ARDS. The reasons why certain patients with LC deteriorate into ALI/ARDS remain unclear. Oxidants, by nature of their ability to affect multiple pathways implicated in hypoxia and inflammation, are believed to bean important factor. Our recent findings show that generation of oxidants is an important cause of permeability injury and acute inflammation in LC.

We have also documented the recruitment and activation of neutrophils and lung tissue macrophages, as well as the production of multiple cytokines and chemokines in LCmice[7]. Chemokines from lung cells stimulate chemotaxis and influence the directional motility of neutrophils[31]. Based on our data presented in this manuscript, MnTBAP protects lung injury and acute inflammation associated with contusion in the rat model. The mechanism of attenuation of inflammation by SOD mimetics is characterized by reduction of peroxynitrite formation through the elimination of superoxide anions before they react with nitric oxide. Recent findings obtained from our laboratory using immunohistochemistry and mass spectrometry revealed that levels of 3-nitrotyrosine were increased markedly at 4 h after LC. Additionally, findings confirmed that consumption of NO due to excess superoxide, resulting in peroxynitrite formation, leads to diminished vascular reactivity afterLC[18].

Lung tissue nitrotyrosine and dityrosine was increased ~2 fold in LC vs. controls (Fig. 7). As shown in Fig. 7, there was a decrease in dityrosine and nitrotyrosine in response to MnTBAP treatment consistent with reduced oxidative stress and permeability. These results demonstrate that excess superoxide (likely from NOX enzymes or mitochondria) from LC leads to both oxidant stress and increased permeability. The precise mechanisms of how a cell-permeable SOD mimetic like MnTBAP works to decrease the inflammation and subsequent permeability injury are a matter of intense interest. Several studies have indicated that the main mechanism of action that attenuates inflammation is through a decrease in peroxynitrite concentrations[32,33]. In contrast, the biologic effects of peroxynitrite are multiple and include an increase in lipid peroxidation, enzyme inactivation, DNA damage, and direct cytotoxicity [32]. MnTBAP has potent protective effects in experimental systems where the injury is likely to be mediated by superoxide alone[22]. Additionally, mice lacking Manganese mitochondrial SOD (*Sod2*tm1cje −/−) had a significant prolongation of their survival when treated with MnTBAP[34–36]. Superoxides in themselves are pro-inflammatory and it is a distinct possibility that the results observed in our study are a direct consequence of a decrease in superoxide concentrations. These results are also supported by previous observations that showed that the use of SOD mimetics was associated with inhibition of several inflammatory cytokines, including interleukin-1β (IL-1β) and IL-6, in models of acute and chronic inflammation [37,38]. Data from our laboratory indicated that the acute inflammatory response in LC is neutrophil-dependent[6,8,25]. Treatment with MnTBAP demonstrated a decrease in the number of neutrophils in the BAL in the MnTBAP rats, which represents a prominent and important finding in the current manuscript. The current study does not preclude the direct effect of MnTBAP in decreasing inflammation independent of the decrease in peroxynitrite. These findings are consistent with the observation that oxidants are responsible for injury and inflammation after LC.

Antioxidant therapy has been used in various models of lung injury and pulmonary hypertension. Administration of intratracheal recombinant human superoxide dismutase (rhSOD) to lambs with pulmonary hypertension was shown to be as effective as inhaled nitric oxide in improving oxygenation and significantly reduced 3-nitrotyrosine formation in

the lungs[39]. There are human studies using similar compounds establishing the safety profile of antioxidants. Davis et al administered intratracheal rhSOD to premature infants and demonstrated reduced need for pulmonary medications in later infancy [40]. Ongoing trials are being planned using manganese porphyrin antioxidants such as AEOL 10150 in amyotrophic lateral sclerosis (ALS). Similar use of antioxidants may ameliorate ALI/ARDS in patients with LC.

In conclusion, the data presented suggest that scavenging superoxide and reducing formation of peroxynitrite attenuate inflammation and vascular permeability in LC animals, suggesting a central role for superoxide in this process. MnTBAP, functioning as a superoxide scavenger, could be an ideal therapeutic agent in trauma patients with LC.

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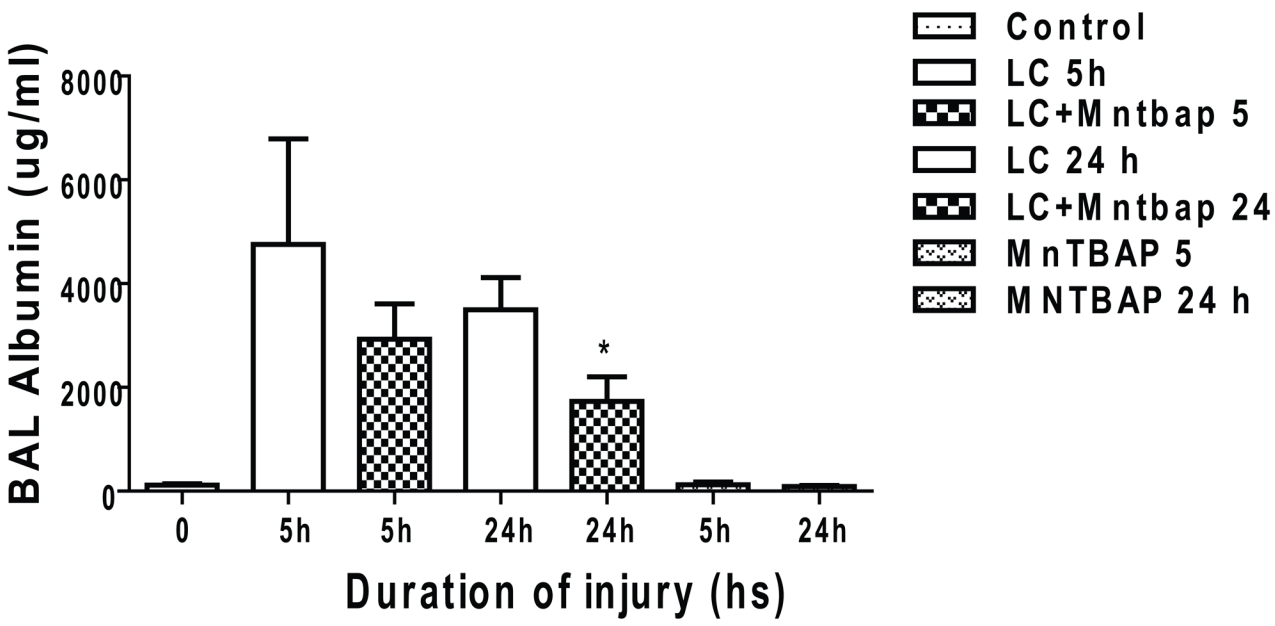


Figure 1. SOD mimetic MnTBAP administration attenuates permeability injury in rats after LC

Rats with appropriate controls (n=12) were subjected to LC and treated with MnTBAP. Animals were examined at 5 and 24 h time points where BAL albumins by ELISA were analyzed. Each experiment was repeated at least three times.*$P < 0.05$ compared to injured animals (LC) at the same time point, as determined by unpaired student t-test with Welch's correction.

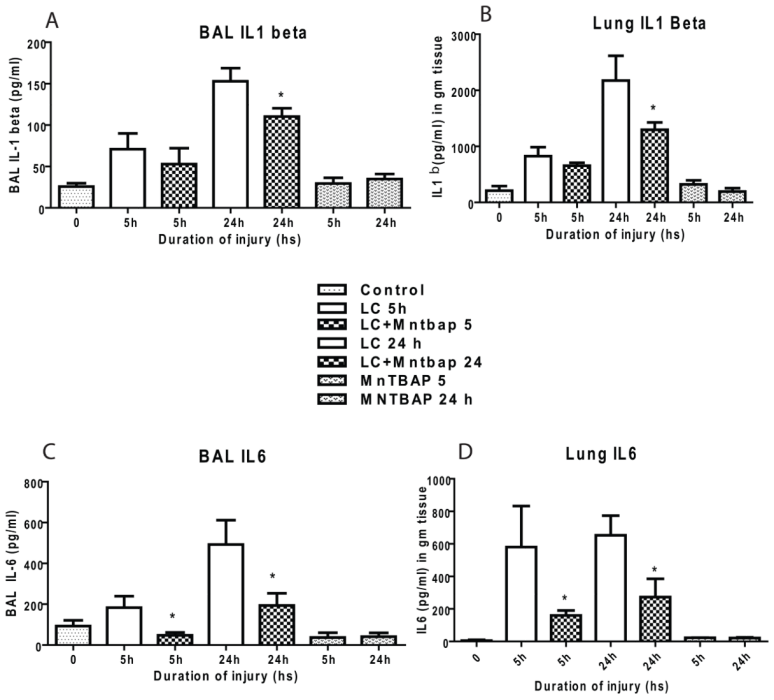


Figure 2. MnTBAP administration reduced the pro-inflammatory cytokines in rats after LC

After LC, rats were sacrificed at 5 and 24 h time points, and the concentrations of albumin in BAL were determined by ELISA: IL-1β (Fig. 2A–B) and IL-6 (Fig. 2C–D). Each experiment was repeated at least three times.*$P < 0.05$ compared to injured animals (LC) at the same time point, as determined by unpaired t-test with Welch's correction.

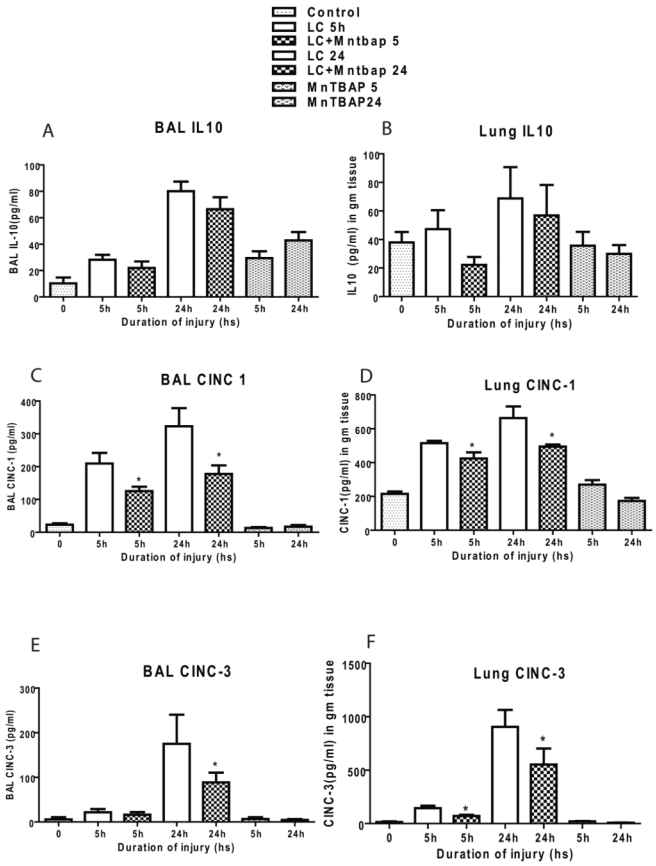


Figure 3. SOD mimetic MnTBAP attenuates lung inflammation in rats after LC

After LC, rats were sacrificed at 5 and 24 h time points, and the concentrations of cytokines in BAL and lungs, IL-10 (3A and B), CINC-1 (3C and D) and CINC-3 (3E and F) were determined by ELISA. Values are represented as mean ± SEM (n=12). Each experiment was repeated at least three times. *P < 0.05 compared to injured animals (LC) at the same time point as determined by unpaired t-test with Welch's correction.

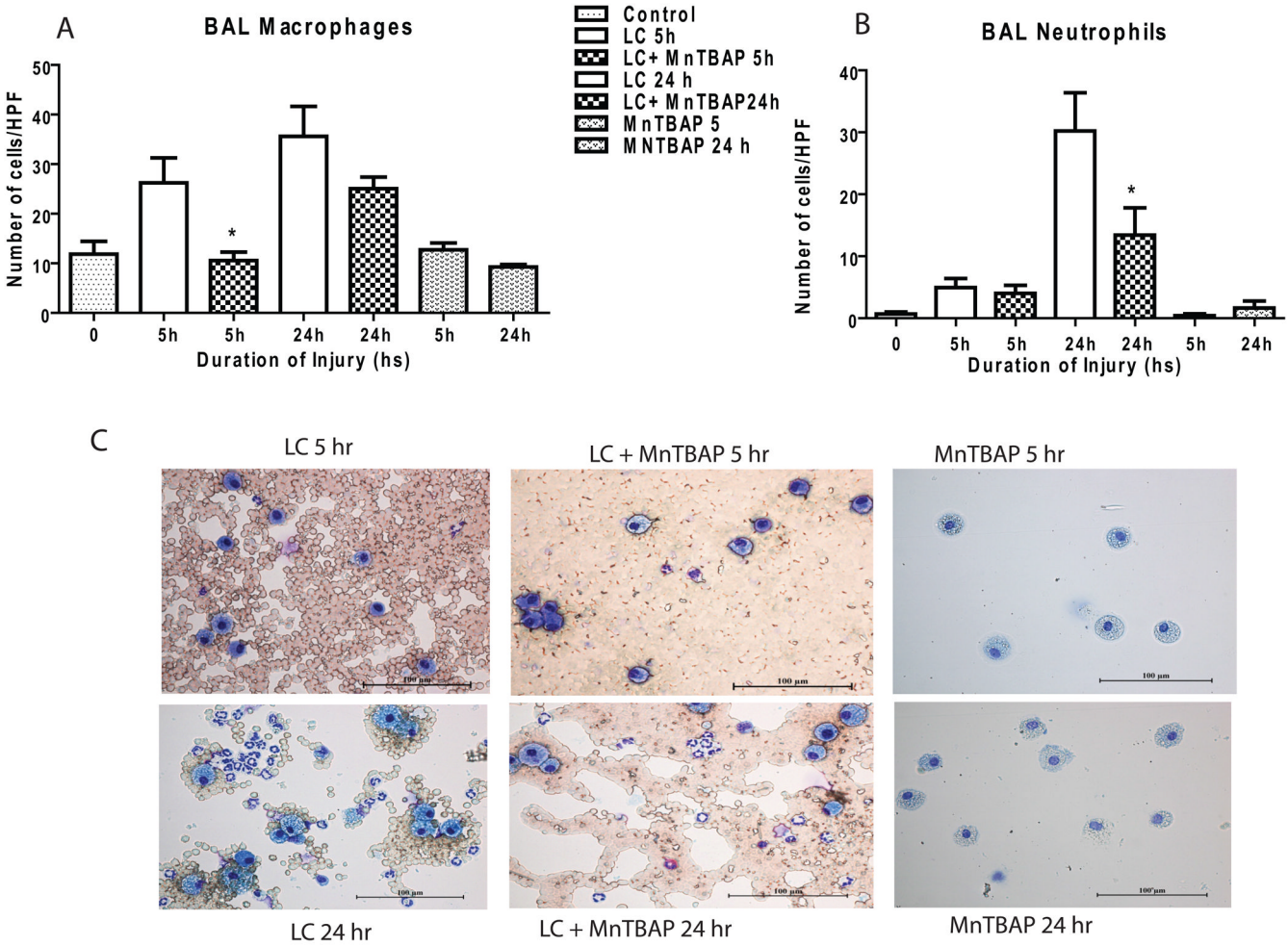


Figure 4. Cellular responses in MnTBAP administered rats after LC

Cytospin analysisof macrophages in BAL samples collected at 5 and 24 h after lung contusion visualized by light microscopy at 200 × magnification (Fig. 4A). MnTBAP treatment significantly attenuates rat neutrophil levels in the BAL *after* LC (Fig. 4B). The representative cytospin photomicrographs of macrophages and neutrophils are shown in Fig. 4C. Values are represented as mean ± SEM (n=12).*P < 0.05 compared to injured animals (LC) at the same time point as determined by unpaired t-test with Welch's correction.

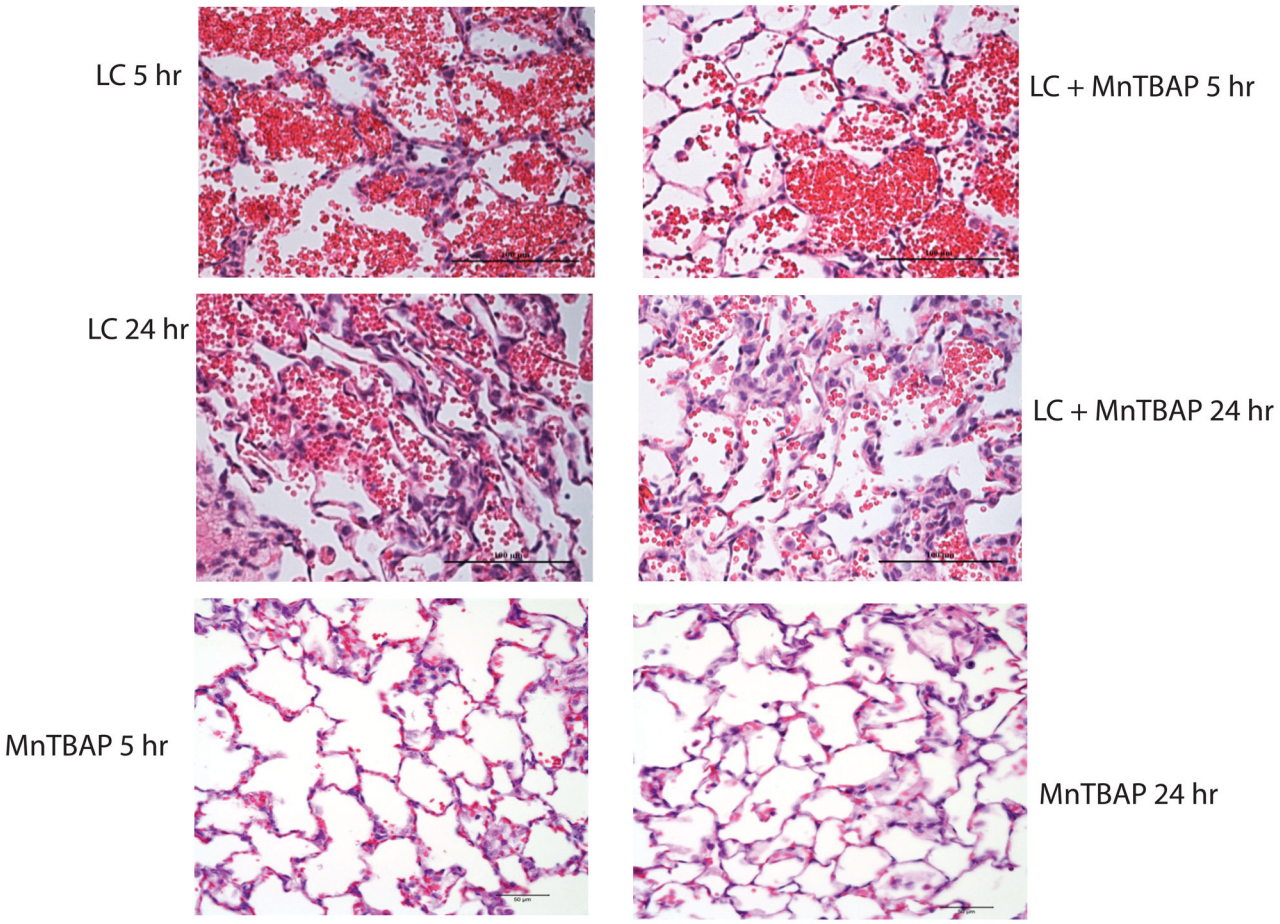
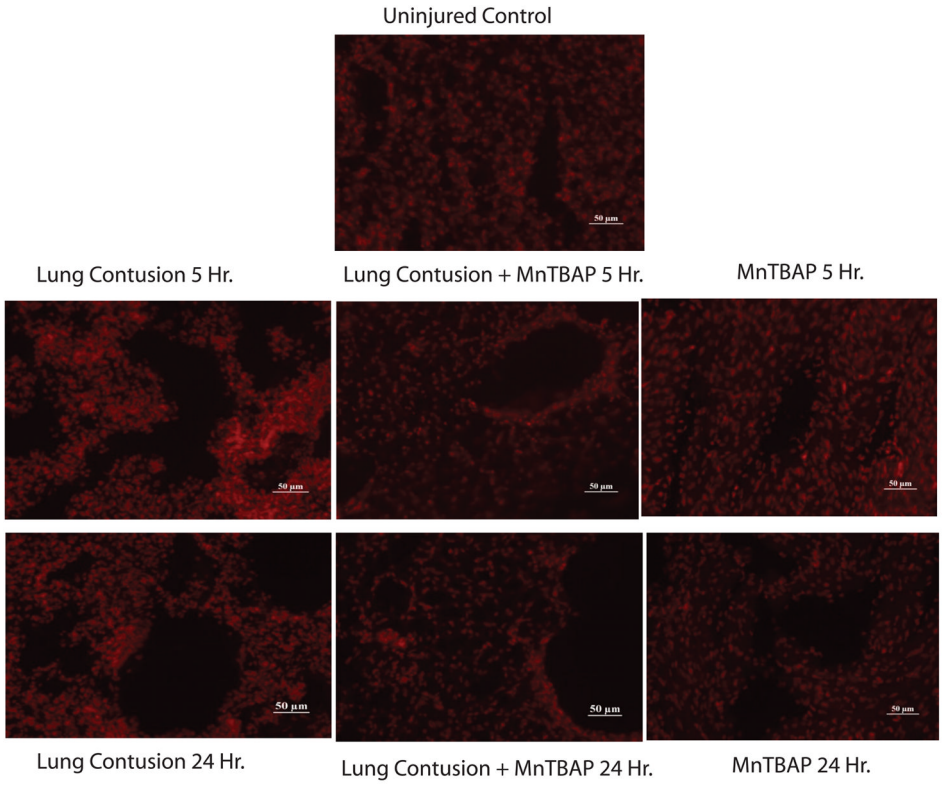


Figure 5. Administration of MnTBAP with LC reduced tissue inflammation and necrosis at 24 hours

Histopathology of lung in rat at 5 and 24 h after lung contusion are shown at 200× magnification. Specimens shown are representative of at least three independent experiments.



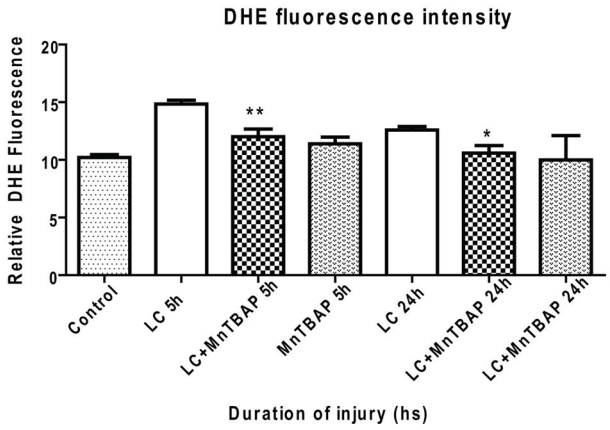


Figure 6. Administration of MnTBAP leads to a reduction in superoxides in lung samples after LC

Fluorescent staining for superoxide anions increases in pulmonary sections *after* LC. There were significant differences observed at the 5 or 24 h time points compared to LC+ MnTBAP. This figure is representative of at least three independent experiments and the fluorescent intensity represents mean ± S.E.M of five different densitometry measurements of each sample.

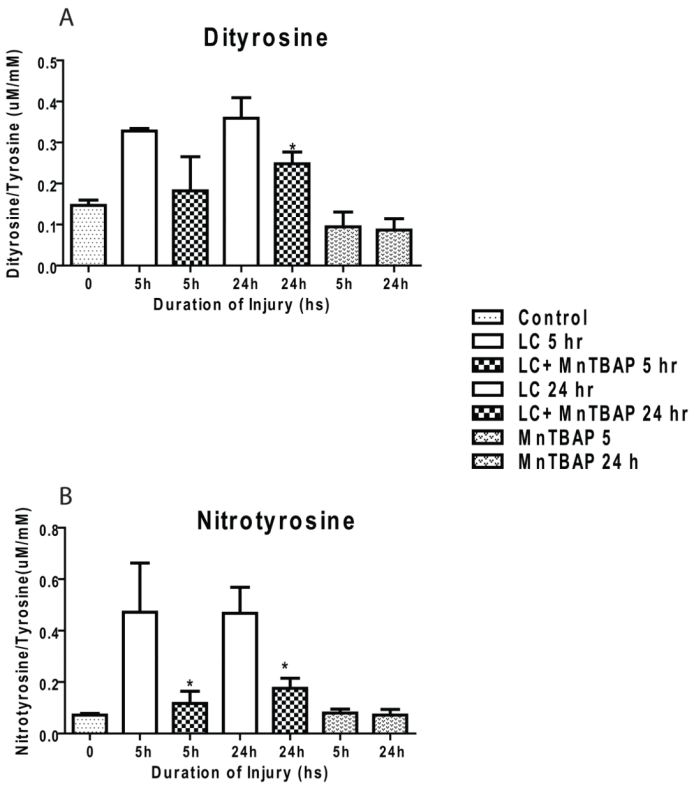


Figure 7. MnTBAP reduces oxidized Tyrosines in rats after LC

LC was induced in rats with and without treatment with MnTBAP. Animals were examined at 5 and 24 h time points and the lung samples were subjected to MS/MS for quantification of protein-bound dityrosine and nitrotyrosine levels (Fig. 7A and B).*$P < 0.05$ compared to corresponding treated animals (LC) at the same time point, as determined by student's t-test.