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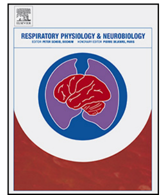

Relationship between neutrophil influx and oxidative stress in alveolar space in lipopolysaccharide-induced lung injury

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ABSTRACT

We intratracheally administered lipopolysaccharide (LPS) to ICR mice and then collected BAL fluid and lung tissue to determine whether levels of neutrophils and/or myeloperoxidase (MPO) in bronchoalveolar lavage (BAL) fluid reflect lung tissue damage. Robust neutrophil accumulation into the alveolar space and lung tissue were almost completely abolished at seven days along with oxidative stress markers in the lung. However, lung injury scores and lung wet/dry ratios, as well as MPO and oxidative stress markers in BAL fluid were significantly increased at five and seven days after LPS administration. At later time points, BAL neutrophils generated more MPO activity and ROS than those harvested sooner after LPS administration. Although elevated neutrophil levels in BAL fluid reflected oxidative stress in the lungs, MPO might serve as a useful marker to evaluate damage sustained by epithelial cells over the long term.
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1. Introduction

Acute respiratory distress syndrome (ARDS) is a type of acute diffuse, inflammatory lung injury that leads to increased pulmonary vascular permeability, increased lung weight, and loss of aerated lung tissue. The clinical hallmarks are hypoxemia and bilateral radiographic opacities associated with increased venous admixture, increased physiological dead space and decreased lung compliance (Force et al., 2012). Acute respiratory distress syndrome is a frequent complication among critically ill patients and it is responsible for high morbidity and mortality rates (Lesur et al., 1999; Ware and Matthay, 2000). Treatment of the underlying disease and supportive care using the "lung protective" strategies of mechanical ventilation and prone positioning, contribute to successful clinical outcomes (TARDS Network, 2000; Guerin et al., 2013). However, specific therapies have not been established and once the cascade of events leading to ARDS has been initiated, the condition becomes much less amenable to specific treatment.

Reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and hypochlorous acid (HOCl) play central roles in the pathogenesis of acute lung injury (Haegens et al., 2009; Tate and Repine, 1983). Endothelial or epithelial cells express several antioxidants such as superoxide dismutase, catalase and glutathione peroxidase to neutralize free radicals and counteract the detrimental effects of ROS (Fink, 2002). However, ROS generated by phagocytes during the acute inflammatory response overwhelm these antioxidants and lead to cell and lung damage. Neutrophils that have high oxidant-generating capacity migrate into the alveolar space where they degranulate and release proteins from azurophilic granules into phagolysosomes (Nauseef, 2001). Bronchoalveolar lavage (BAL) is a diagnostic method of sampling cells in the airway-alveolar space and soluble substances in the extracellular lining. The number of neutrophils in BAL fluid is robustly increased in ARDS, and the ratios (%) of neutrophils are markers of disease activity (Steinberg and Hudson, 1994). The time course of transpulmonary polymorphonuclear leukocyte migration has been investigated (Hirano, 1997; Reutershan et al., 2005). However, whether or not inflammatory cells, especially neutrophils that presently serve as clinical markers in BAL fluid, reflect the extent of damage in lung tissues remains obscure.

The short biological half-life of ROS renders them difficult to measure directly in biological materials from the lungs of patients with ARDS, and reports describing increased ROS activity in ARDS are scant (Baldwin et al., 1986). Alternatively, the oxidative modification of ROS targets such as proteins, lipids, and antioxidants are regarded as useful markers with which to indirectly reflect oxidative stress. Levels of protein carbonyls, myeloperoxidase (MPO), thiobarbituric acid-reactive substances (TBARS), lipid oxidation products and oxidized glutathione are elevated in BAL fluid from patients with ARDS (Bunnell and Pacht, 1993; Winterbourn et al., 2000). Whether or not oxidative stress markers exactly reflect lung

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oxidative stress in patients with ARDS is unknown. These oxidative markers have been evaluated in animal models of ARDS to determine the amount of oxidative stress in the lungs. However, few studies have investigated the same oxidative stress markers both in BAL fluid and in lung tissue (Bergeron et al., 1998).

The present study investigated whether or not neutrophils and MPO in BAL fluid can reflect oxidative stress or epithelial damage in the lungs of a mouse model of LPS-induced lung injury. We compared the kinetics of various oxidative stress markers with neutrophil accumulation and MPO activities in BAL fluid and tissues from mouse lungs with lipopolysaccharide (LPS)-induced lung injury. We also examined the ROS-producing potential of neutrophils harvested from BAL at various intervals after the intratracheal instillation of LPS to produce ROS. Not only a higher ratio of neutrophils but also an increase in MPO activity in BAL fluid suggested the existence of epithelial cell damage and oxidative stress both in BAL fluid and in the lungs with LPS-induced lung injury. Thus, MPO might be a useful marker to evaluate long term damage sustained by epithelial cells.

2. Materials and methods

2.1. Animals

Nine-week-old male ICR mice purchased from Japan Clea (Tokyo, Japan) were housed in plastic chambers with free access to food and water. None of the mice had gross pathological lesions. The Ethics Committee for Animal Research at Hokkaido University School of Medicine approved the experimental protocols.

2.2. Mouse model of LPS-induced lung injury

Saline (50 μL) containing 200 μg of LPS (Sigma Chemical Co., St. Louis, MO, USA) was intratracheally administered to mice anesthetized with a mixture of ketamine and xylazine as described (Betsuyaku et al., 1999; Ito et al., 2009). Age-matched, untreated healthy mice served as controls.

2.3. BAL and tissue measurements

2.3.1. Wet/dry weight ratio

The wet lungs of mice from which BAL had not been collected were weighed immediately after dissection, dried at 37 °C for 72 h, and then weighed once again to determine the wet/dry (W/D) weight ratio.

2.3.2. Lung histopathology

Paraffin-embedded lung sections were stained with hematoxylin and eosin for assessment by light microscopy. Lung damage was graded from 0 (normal) to 4 (severe) based on the criteria of interstitial inflammation, neutrophil infiltration, congestion and edema (Michetti et al., 2003). Lung damage was scored by adding the individual scores for each category and the score for each mouse was calculated as the mean of four lung sections.

2.3.3. BAL and sampling of mouse lung tissues

Mice were killed by CO_2 narcosis at 1, 3, 5, 7 and 14 days after LPS injection ($n = 5$–6 per time point) and BAL was collected using three 0.6-mL injections of saline through a tracheal cannula. Red blood cells in BAL fluid samples were disrupted using red blood cell lysis buffer (Sigma) and then total numbers of cells were counted using a hemocytometer. Cell differentials in BAL fluid were examined in Cytospin preparations stained with Diff-Quik reagent (Sysmex International Reagents, Kobe, Japan). After BAL fluid was collected, the lungs were inflated with 50% (v/v) Tissue-Tek OCT (Sakura Finetek USA, Torrance, CA, USA) in RNase-free phosphate-buffered saline (PBS) containing 10% sucrose and stored at −80 °C as described (Suzuki et al., 2008).

2.3.4. Immunohistochemical evaluation of neutrophils in lungs

Lung sections were immunostained for Gr-1 as described (Moriyama et al., 2010). Briefly, non-specific binding was blocked for 30 min using 5% (v/v) normal rabbit serum in PBS. Neutrophils were detected using a polyclonal rat anti Ly-6G (Gr-1) monoclonal antibody (BD Biosciences, San Jose, CA, USA) followed by anti-rat IgG-horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). Labeling was visualized using diaminobenzidine as the chromogen (Vector Laboratories). Gr-1-positive cells were counted in five random fields per section of 5–6 grafts per group, and then the ratio (%) of total cells per high-power field was calculated.

2.3.5. Assay of MPO activity

We spectrophotometrically assayed MPO activity in BAL fluid and lung tissues as described (Haslam and Baughman, 1999). Briefly, BAL fluid (25 μL) or lung homogenate was reacted with H_2O_2 (0.0005%) in the presence of o-dianisidine dihydrochloride (0.167 mg/mL) for 30 min and changes in absorbance at 450 nm were measured. Protein concentrations of tissue extracts were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

2.3.6. Assessment of carbonylated protein in BAL fluid

The carbonylation of proteins in BAL fluid was measured by Western blotting as described (Nagai et al., 2006, 2008). Briefly, raw BAL fluid (16 μL) was derivatized with dinitrophenylhydrazine (DNP) using the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Temecula, CA, USA) and resolved by electrophoresis on 10% SDS–polyacrylamide gels. Proteins were Western blotted with anti-DNP antibody and band intensity was calculated using NIH Image software (version 1.62). The intensity of the 68-kDa band corresponding to carbonylated albumin on each blot is shown as arbitrary units (AU).

2.3.7. Total protein assay

Total protein concentration in BAL fluid was quantified using the bicinchoninic acid microassay method (Pierce Chemical).

2.3.8. Measurement of LPO, GSH and GSSG in BAL fluid

Levels of LPO, GSH and GSSG in BAL fluid were measured using kits according to the manufacturer's protocols (Cayman Chemical, Ann Arbor, MI, USA).

2.3.9. Measurement of protein carbonyl contents of the lung

Protein carbonyl contents in lung homogenates were determined using a protein carbonyl assay kit (Cayman Chemical), according to the manufacturer's instructions.

2.3.10. Immunohistochemical evaluation of 4 hydroxy-2-nonenal modified proteins (4-HNE)

Frozen sections cut at 4 μm were fixed in 4% paraformaldehyde for 10 min and then immunostained using the Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA, USA) with rabbit anti-4-HNE (Alpha Diagnostic, San Antonio, TX, USA) antibody. Non-specific binding was blocked for 1 h using 5% goat serum diluted in PBS at room temperature, and then the sections were incubated in primary antibody (diluted 1:3000) at room temperature for 30 min. Biotinylated universal secondary antibody and Elite ABC reagent were applied at room temperature for 30 min. The sections were washed with Tris-buffered saline containing 0.05% Tween 20 (Sigma) and then alkaline phosphatase substrate was

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