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Original Contribution

The effects of aging on pulmonary oxidative damage, protein nitration, and extracellular superoxide dismutase down-regulation during systemic inflammation

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ABSTRACT

Systemic inflammatory response syndrome (SIRS), a serious clinical condition characterized by whole-body inflammation, is particularly threatening for elderly patients, who suffer much higher mortality rates than the young. A major pathological consequence of SIRS is acute lung injury caused by neutrophil-mediated oxidative damage. Previously, we reported an increase in protein tyrosine nitration (a marker of oxidative/nitrosative damage) and a decrease in the antioxidant enzyme extracellular superoxide dismutase (EC-SOD) in the lungs of young mice during endotoxemia-induced SIRS. Here we demonstrate that during endotoxemia, down-regulation of EC-SOD is significantly more profound and prolonged, whereas up-regulation of iNOS is augmented, in aged compared to young mice. Aged mice also showed 2.5-fold higher protein nitration levels, compared to young mice, with particularly strong nitration in the pulmonary vascular endothelium during SIRS. Additionally, by two-dimensional gel electrophoresis, Western blotting, and mass spectrometry, we identified proteins that show increased tyrosine nitration in age- and SIRS-dependent manners; these proteins (profilin-1, transgelin-2, LASP 1, tropomyosin, and myosin) include components of the actin cytoskeleton responsible for maintaining pulmonary vascular permeability. Reduced EC-SOD in combination with increased oxidative/nitrosative damage and altered cytoskeletal protein function due to tyrosine nitration may contribute to augmented lung injury in the aged with SIRS.

Aging is associated with reduced stress tolerance. Vulnerability to various physiological stresses such as infection, inflammation, and oxidative damage increases with age and is causally related to clinical problems in the elderly. Sepsis is an infection-initiated clinical condition characterized by systemic inflammation [1–3]. The progression of sepsis occurs by a loss of homeostasis, characterized by uncontrolled inflammation, oxidative damage to the vascular endothelium, and intravascular coagulation. These conditions lead to multiple organ failure and death in a large number of patients [4]. Sepsis is a particularly serious problem in the geriatric population, as the elderly exhibit significantly elevated mortality. Earlier studies noted 30–40% mortality among the elderly with sepsis, compared to 4–5% for younger patients [5,6]. More recent analyses estimate that more than 700,000 patients present with sepsis yearly in the United States, resulting in 215,000 deaths, of which the majority are elderly people [7,8]. Although age-related dysregulation of the inflammatory response and thrombosis seem to be involved [9–11], the precise mechanisms for the age-dependent vulnerability to sepsis remain unclear.

One of the major pathological consequences of the systemic inflammatory response syndrome (SIRS) is acute lung injury. Oxidative damage due to the production of superoxide during SIRS is highly associated with acute lung injury [12] although a causal relationship still has to be definitively established. 3-Nitrotyrosine is a known biomarker of nitric oxide ($^{\bullet}NO$)-dependent oxidative/nitrosative damage [13–15]. Recently we demonstrated that protein tyrosine nitration is increased in the lungs of young mice during periods of lipopolysaccharide (LPS)-induced systemic inflammation [16]. During the inflammatory response, neutrophils undergo a respiratory burst and produce superoxide ($O_2^{\bullet-}$). The superoxide anion reacts with $^{\bullet}NO$ (generated by inducible NO synthase (iNOS) during the immune response) and produces peroxynitrite ($ONOO^-$), a toxic oxidant and nitrating agent. Breakdown of peroxynitrite produces highly toxic reactive nitrogen and oxygen species such as $\cdot NO_2$ and $^{\bullet}OH$ and $CO_3^{\bullet-}$. These radicals promote the posttranslational modification of tyrosine to 3-nitrotyrosine, which alters the structure and conformation of target proteins and can compromise their function [17]. The presence of nitrated proteins has been implicated in many disease states; however, the identification of these proteins has only begun [18].

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Although reactive oxygen species (ROS) have physiologically essential roles in host defense, overproduction of ROS during SIRS is highly toxic to host tissues, resulting in severe pathophysiological consequences such as vascular endothelial damage [19–21]. In a protective mechanism, superoxide is converted by antioxidant enzymes, superoxide dismutases (SOD), to form hydrogen peroxide (H_2O_2), which is further converted to H_2O and O_2 by another antioxidant enzyme, catalase [22]. There are three different mammalian SODs: intracellular copper–zinc SOD (Cu/Zn-SOD or SOD1), mitochondrial manganese SOD (Mn-SOD or SOD2), and extracellular SOD (EC-SOD or SOD3). EC-SOD is the predominant antioxidant enzyme that is localized to the extracellular space [23–28]. It is strongly expressed in vascular smooth muscle cells, alveolar type II cells, and lung macrophages, among other cell types [29], and localized throughout the blood vessel wall; the major source of the EC-SOD protein is thought to be vascular muscle and not endothelium [30]. An in situ hybridization study in mice demonstrated no mRNA expression of EC-SOD on the capillary wall, suggesting that EC-SOD accumulates in the endothelial cells after injury [31]. In our previous study we demonstrated that EC-SOD levels, but not those of Cu/Zn- or Mn-SOD, are decreased in the lungs during systemic inflammation. Additionally, using EC-SOD transgenic mice, we determined that this enzyme plays a protective role against inflammation-mediated oxidative/nitrosative damage and mortality [16].

In this study, we show that the levels of EC-SOD, but not other SODs, decrease in the lungs of both young and aged mice during systemic inflammation and that the decrease is significantly more profound in the aged. Furthermore, we show that protein tyrosine nitration is significantly elevated in the lungs of aged mice compared to young mice and we identified several of the nitrated proteins whose loss of function may contribute to increased lung injury and vascular permeability in the aged during sepsis.

Materials and methods

Animals

Young (4–6 months of age) and aged (24–26 months of age) male C57BL/6 mice were obtained from colonies of the National Institute on Aging (Bethesda, MD, USA). Mice were acclimated for at least 14 days in a 12:12-h light:dark cycle with free access to water and regular chow diet (LabDiet, Brentwood, MO, USA) before experiments began and throughout each experiment. All animal care and surgical procedures were approved by the Institutional Animal Care and Use Committees at the University of Texas Medical Branch and the University of Kentucky.

LPS model for endotoxemia

Acute systemic inflammation was induced by intraperitoneal (ip) injection with bacterial endotoxin LPS (2.5 mg/kg) derived from *Pseudomonas aeruginosa* (Sigma Chemical, St. Louis, MO, USA). For tissue protein or RNA analyses, mice were anesthetized with isoflurane inhalation, the inferior vena cava was cut, and the entire vasculature was perfused with physiological saline through the cardiac ventricles as previously described [16]. Lungs were harvested 0, 6, and 12 h after LPS injection and flash-frozen in liquid nitrogen. Major organs from dead mice were examined, and animals with any evident signs of tumor were excluded from the study.

Histological analysis and immunohistochemistry

Endotoxic and control mice were anesthetized with isoflurane inhalation, and the lungs were filled with formalin from the trachea (to maintain the organ structure), harvested, and fixed in 10% neutral-buffered formalin. The harvested tissues were embedded in paraffin, and sections (5 μm) were cut from paraffin blocks and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed using the Dako Cytomation En Vision + System-HRP (Dako, Glostrup, Denmark). Sections were stained with 1:1000 diluted anti-nitrotyrosine antibody (Millipore, Billerica, MA, USA) at 4 °C overnight and counterstained with hematoxylin. Photomicrographs were taken using a Leica DFC295 digital camera and Nikon Eclipse E200 microscope with Leica Application Suite version 3.5.0 (100× magnification).

Protein extraction, 1D gel electrophoresis, and Western blot analysis

Frozen lung tissues were homogenized in an SDS-based lysis buffer; protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA) and equal amounts of protein (40 μg) were resolved on Nu-PAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and electrophoretically transferred to polyvinylidene difluoride membranes [16]. The membranes were incubated with anti-EC-SOD [16], anti-Cu/Zn-SOD, and anti-Mn-SOD (Stressgen, Victoria, BC, Canada) antibodies as previously described [16]. For nitrotyrosine detection, proteins were transferred to nitrocellulose membranes and incubated with a horseradish peroxidase (HRP)-conjugated antibody against nitrotyrosine as previously described [16]. The membranes were washed and the immunoreaction was visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL, USA). All membranes were reprobed with anti-β-actin antibody (Sigma Chemical) to confirm equal loading of the protein samples. The intensity of each autoradiograph was determined by densitometric analysis (Kodak 1D software; Carestream Health, Rochester, NY, USA) and the level of each protein was normalized to β-actin.

RNA analysis

Total RNA was isolated from tissues using guanidine/phenol solution as previously described [32]. The RNA samples (20 μg each) were subjected to Northern blot analysis as recently described [16]. To prepare probe DNA, an EC-SOD cDNA fragment (264 bp) was amplified by reverse transcriptase–polymerase chain reaction from mouse lung total RNA [16], and a HincII–EcoRI fragment (817 bp) of mouse iNOS cDNA was obtained by a restriction digestion of plasmid piNOSL3, which was kindly provided by Dr. Regino Perez-Polo at the University of Texas Medical Branch.

Protein extraction, 2D gel electrophoresis, and Western blot analysis

Frozen lung tissues were homogenized in a urea-based lysis buffer (Bio-Rad) and the protein concentrations determined by RC DC protein assay (Bio-Rad). Two-hundred-microgram protein samples were prepared by pooling lung homogenates from four mice. Isoelectric focusing was performed in the first dimension using 11-cm, pH 3–10 immobilized pH gradient (IPG) strips. In the second dimension, IPG strips were equilibrated with TCEP and iodoacetamide. The strips were then embedded in agarose above 8–16% Tris–HCl gels and run at 150 V for 2.5 h. Proteins were electrophoretically transferred to nitrocellulose membranes and incubated with an HRP-conjugated antibody against nitrotyrosine. The membranes were washed and the immunoreaction was visualized by ECL. Exposed film was imaged on a ProSCAN ProXPRESS 2D proteomic imaging system (PerkinElmer, Waltham, MA, USA) for analysis.

Gel analyses and spot excision

Gels were fixed in 10% methanol, 7% acetic acid, and stained with SYPRO ruby (Invitrogen), destained in 10% ethanol, and imaged on the ProSCAN ProXPRESS 2D proteomic imaging system. A total of eight 2D gel images and eight Western blots (two from each treatment group)