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Original Contribution

Redox regulation of mitophagy in the lung during murine *Staphylococcus aureus* sepsis

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ABSTRACT

Oxidative mitochondrial damage is closely linked to inflammation and cell death, but low levels of reactive oxygen and nitrogen species serve as signals that involve mitochondrial repair and resolution of inflammation. More specifically, cytoprotection relies on the elimination of damaged mitochondria by selective autophagy (mitophagy) during mitochondrial quality control. This aim of this study was to identify and localize mitophagy in the mouse lung as a potentially upregulatable redox response to *Staphylococcus aureus* sepsis. Fibrin clots loaded with *S. aureus* (1×10^7 CFU) were implanted abdominally into anesthetized C57BL/6 and B6.129X1-*Nfe2l2tm1Ywk*/J (*Nrf2*$^{-/-}$) mice. At the time of implantation, mice were given vancomycin (6 mg/kg) and fluid resuscitation. Mouse lungs were harvested at 0, 6, 24, and 48 h for bronchoalveolar lavage (BAL), Western blot analysis, and qRT-PCR. To localize mitochondria with autophagy protein LC3, we used lung immunofluorescence staining in LC3–GFP transgenic mice. In C57BL/6 mice, sepsis-induced pulmonary inflammation was detected by significant increases in mRNA for the inflammatory markers IL-1β and TNF-α at 6 and 24 h, respectively. BAL cell count and protein also increased. Sepsis suppressed lung Beclin-1 protein, but not mRNA, suggesting activation of canonical autophagy. Notably sepsis also increased the LC3-II autophagosome marker, as well as the lung's noncanonical autophagy pathway as evidenced by loss of p62, a redox-regulated scaffolding protein of the autophagosome. In LC3–GFP mouse lungs, immunofluorescence staining showed colocalization of LC3-II to mitochondria, mainly in type 2 epithelium and alveolar macrophages. In contrast, marked accumulation of p62, as well as attenuation of LC3-II in *Nrf2*-knockout mice supported an overall decrease in autophagic turnover. The downregulation of canonical autophagy during sepsis may contribute to lung inflammation, whereas the switch to noncanonical autophagy selectively removes damaged mitochondria and accompanies tissue repair and cell survival. Furthermore, mitophagy in the alveolar region appears to depend on activation of Nrf2. Thus, efforts to promote mitophagy may be a useful therapeutic adjunct for acute lung injury in sepsis.

Sepsis is now the leading cause of death in critically ill patients [1] and 40% of these patients develop acute lung injury (ALI), which is characterized by inflammatory cell infiltration and loss of type I alveolar epithelium. This leads to pulmonary capillary leak and acute respiratory failure [2]. ALI is also accompanied by severe inflammation and associated with oxidative stress caused by excessive reactive oxygen and nitrogen species (ROS/RNS) production [3]. ROS/RNS at low levels serve as signaling molecules to activate redox-sensitive genes [4], but uncontrolled ROS generation eventually overwhelms cells, causing structural damage, particularly to mitochondria. More specifically, this excessive ROS production directly inhibits oxidative phosphorylation [5]. Furthermore, loss of mitochondrial function results in epithelial apoptosis, causing epithelial barrier breakdown and ALI [6]. The resolution of mitochondrial oxidative stress is linked directly to survival in severe sepsis [7].

Mitochondrial-derived reactive oxygen species (mROS) are produced normally as by-products of oxidative phosphorylation; however, inflammation leads to excessive mROS efflux that damages mitochondria [8,9]. Damaged mitochondria are a primary source of cellular oxidative stress. In response, nuclear-encoded mitochondrial gene expression is induced to match mitochondrial function with cellular energy demands [10]. Moreover, oxidative mitochondrial

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damage results in activation of genes that contain antioxidant-response elements (AREs) that contribute to both mitochondrial antioxidant defense and mitochondrial quality control [11].

Mitochondrial quality control during oxidative stress requires a key transcription factor, Nrf2 (nuclear factor E2-related factor 2), which is responsible for the expression of superoxide dismutase 2 (SOD2) and more than 100 other cytoprotective genes [12]. Nfe2l2/Nrf2 is part of the major cellular defenses against oxidative stress after nuclear localization and transcription of ARE-containing genes by small Maf proteins [13,14]. Nrf2 is normally sequestered in the cytosol by Keap1 (Kelch-like ECH-associating protein 1), which results in its ubiquitination and degradation by the 26S proteasome [15]. During oxidant stress, cysteine residues on Keap1 are oxidized, and this allows Nrf2 to translocate to the nucleus and to transcribe ARE-containing genes including selected mitochondrial quality control genes [16].

Mitochondrial quality control also involves the clearance of irreparably damaged mitochondria via the process of selective macroautophagy or mitophagy [17]. These damaged mitochondria are segregated from the functional mitochondrial network, engulfed in autophagosomes, and then degraded by lysosomal proteases [18].

Current literature on mitophagy has suggested that autophagy may select mitochondria for removal through either Atg-dependent or Atg-independent pathways [19,20]. Mitophagy is a normal physiological process [21], but is also found in aging [22], as well as in pathophysiological states such as sepsis [23]. Mitophagy in sepsis has been studied previously in the liver [24,25], and autophagy occurs in the lung in ALI [26], but mitophagy has not been characterized in the lung in sepsis.

The purpose of this investigation was to test the hypothesis that *Staphylococcus aureus* sepsis in murine peritonitis induces sufficient oxidative stress to damage parenchymal lung mitochondria and that these mitochondria are removed by mitophagy, which, in conjunction with mitochondrial biogenesis, allows restoration of the lung's oxidative balance during sepsis resolution.

Methods

Materials

Antibodies were purchased as follows: heme oxygenase 1 (HO-1; Assay Designs), Beclin-1 (Cell Signaling), Nrf2 (Santa Cruz), p62 (Abcam), LC3 (Sigma–Aldrich), SOD2 (Abcam), 8-hydroxy-2-deoxyguanosine (8-OHdG; Genetex). Primers used for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) were all obtained from Life Technologies (Atg5, Atg12, Beclin-1, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), SOD2, HO-1). Bafilomycin A1 (BFA) was purchased from LC Laboratories.

Single-stranded DNA (ssDNA) probe

The ssDNA probe used was obtained from Millipore (MAB3299) and has been used in many previous experiments in multiple tissue types to detect apoptotic cells by immunohistochemistry [27–30].

Mice

The mouse studies were preapproved by the Duke IACUC. C57BL/6J wild-type (WT) as well as B6.129X1-*Nfe2l2tm1Ywk*/J (*Nrf2*$^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *S. aureus* (clinical isolate; ATCC 25923) was prepared, counted, and embedded in fibrin clots before being implanted surgically in the abdomen [7]. Wild-type C57BL/6 mice received 1×10^7 colony-forming units (CFU), whereas *Nrf2*$^{-/-}$ mice received 5×10^6 CFU because of their increased sensitivity to sepsis. Mouse lungs were harvested at indicated times for protein and RNA and stored at −80 °C.

LC3–green fluorescent protein (GFP) reporter mice

The mice were purchased from the RIKEN Bioresource center in Japan (RBRC00806). A transgenic vector containing an enhanced LC3–GFP was inserted between a CAG promoter and the SV40 late polyadenylation signal. The GFP is fused to the N-terminus so as not to affect phosphatidylethanolamine conjugation [31]. These mice were also implanted with infected abdominal clots and the lungs were harvested and inflation-fixed in 10% formalin for immunofluorescence microscopy.

Real-time polymerase chain reaction

qRT-PCR was performed with TaqMan primers on StepOnePlus (Applied Biosystems). 18 s rRNA was used as endogenous control. Gene expression was determined by relative quantification.

Protein methods

Fresh lung tissue was homogenized in RIPA buffer and then sonicated on ice. The samples were then placed in a Laemmli buffer. Protein content was measured with bicinchoninic acid using bovine serum albumin (BSA) standards. For Western blotting, protein was resolved by gradient and/or nongradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4–20% or 8%), transferred to an Immobilon P membrane, blocked with 5% nonfat milk, and incubated at 4 °C overnight with polyclonal anti-HO-1 (Enzo, ADI-SPA-895-D), Beclin-1 (Cell Signaling 3738S), Nrf2, p62 (Abcam, ab91526), or LC3α/β (all 1:1000). The membranes were washed in Tris-buffered saline/Tween (TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz at 1:5000). The membranes were rewashed and developed with chemiluminescence reagents. Digitized images were quantified in the middynamic range using ImageJ software. Quantified data were analyzed and graphed and presented in GraphPad Prism 6.

Immunofluorescence microscopy

Lung samples from WT and LC3–GFP were inflation-fixed in formalin and embedded in paraffin, cut into 5-μm slices, mounted on slides, and probed with anti-citrate synthase (GeneTex GTX110624 1:100) and anti-SOD2 (Abcam 13533 1:100). The slides were incubated in secondary goat anti-rabbit antibody conjugated to Alexa 594 (Invitrogen) or Alexa 488 (Invitrogen). For ssDNA, sections were deparaffinized and incubated in 50% formamide at 60 °C for 30 min. Nonspecific binding to primary antibodies was blocked by incubation in 0.1% BSA for 15 min. Then the sections were incubated with anti-ssDNA polyclonal antibody (Dako, diluted at 1:100) overnight. An additional wash in 0.05 M TBS followed, and sections were incubated with Alexa 594 immunoglobulin M diluted at 1:500 for 30 min. Sections were rinsed three times with 0.05 M TBS for 5 min. The nuclei were counterstained with DAPI purchased from Molecular Probes. The slides were observed and images captured under a Nikon Eclipse 50i fluorescence microscope.

For in situ oxidative damage studies, the anti-8-OHdG antibody (Genetex GTX10802) was used. Alexa Fluor 488-labeled secondary antibodies were used to develop the signal for oxidized nucleic acids. Photomicrographs were taken on a Nikon E100 (Nikon Instruments, Melville, NY, USA) microscope at 400× and the nuclear (DAPI blue) and cytoplasmic stained (green) cells were