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Mitochondria-targeted drugs enhance Nlrp3 inflammasome-dependent IL-1β secretion in association with alterations in cellular redox and energy status

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ABSTRACT

The Nlrp3 inflammasome is activated in response to an array of environmental and endogenous molecules leading to caspase-1-dependent IL-1β processing and secretion by myeloid cells. Several identified Nlrp3 inflammasome activators also trigger reactive oxygen species (ROS) production. However, the initial concept that NADPH oxidases are the primary source of ROS production during inflammasome activation is becoming less accepted. Therefore, the importance of mitochondria-derived ROS has been recently explored. In this study, we explore the impact of mitochondria dysfunction and ROS production on Nlrp3 inflammasome stimulation and IL-1β secretion induced by serum amyloid A (SAA) in primary mouse peritoneal macrophages. To induce mitochondrial dysfunction, we utilized antimycin A, which blocks electron flow at complex III, and carbonyl cyanide -*p*-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial oxidative phosphorylation uncoupler. We also utilized a superoxide dismutase mimetic, MnTBAP, which targets the mitochondria, as well as the broad-spectrum antioxidants DPI (diphenyleneiodonium chloride) and ebselen. Our findings demonstrate that SAA alone induces mitochondrial ROS in a time-dependent manner. We observed that MnTBAP and ebselen blocked IL-1β secretion caused by SAA only when added before stimulation, and DPI augmented IL-1β secretion. Surprisingly, these effects were not directly related to intracellular or mitochondrial ROS levels. We also found that mitochondria-targeted drugs increased IL-1β secretion regardless of their impact on mitochondrial function and ROS levels, suggesting that mitochondrial ROS-dependent and -independent mechanisms play a role in the Nlrp3 inflammasome/IL-1β secretion axis in SAA-stimulated cells. Finally, we found that FCCP significantly sustained the association of the Nlrp3 inflammasome complex, which could explain the most robust effect among the drugs tested in enhancing IL-1β secretion in SAA-treated cells. Overall, our data suggest that the Nlrp3 inflammasome/IL-1β secretion axis is a very highly regulated inflammatory pathway that is susceptible not only to changes in mitochondrial or intracellular ROS, but also to changes in overall mitochondrial function.

The interleukin 1 (IL-1) family of cytokines is critical to the host response to infection, participating not only in the acute-phase response from the liver, but also in alterations of metabolism, induction of fever, and leukocyte activation [1]. Overproduction of IL-1β, in particular, is thought to be responsible for a variety of autoinflammatory syndromes, including familial Mediterranean fever and Muckle–Wells syndrome, and is also a contributing factor in rheumatoid arthritis, gout, multiple sclerosis, Alzheimer disease, and diabetes [2–7]. IL-1β is also a pathogenic mediator in several pulmonary disorders, including infection, asthma, ALI/ARDS, transplant rejection, COPD, PAH, sarcoidosis, asbestosis, and silicosis [8–10]. Setting IL-1β apart from other acute-phase cytokines such as IL-6 and TNF-α is the requirement for processing from an inactive proform to an active secreted form by caspase-1-dependent cleavage.

The assembly of a cytoplasmic inflammasome complex facilitates the formation of a molecular platform for caspase-1-dependent secretion of IL-1β. Nlrp3 is a component of inflammasomes that are activated in response to an array of environmental and endogenous molecules [11]. It has been suggested that the mechanism of IL-1β processing and secretion requires sequential steps. The initial event necessary is the synthesis and accumulation of the precursor proteins, including pro-IL-1β and Nlrp3 ("signal 1"), accomplished by several stimuli. These include microbial products such as pathogen-associated molecular pattern molecules, which trigger pathogen recognition receptors (PRRs) such as Toll-like receptors. After PRR triggering, Nlrp3

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activation leads to recruitment of the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and the enzyme caspase-1 to form the Nlrp3 inflammasome complex ("signal 2"), which ultimately is responsible for the cleavage and secretion of IL-1β [12]. In addition, the cleavage and secretion of IL-1β can be enhanced by release of endogenous ATP that stimulates the purinergic receptor P2X7 [13]. Interestingly, several identified Nlrp3 inflammasome activators also trigger reactive oxygen species (ROS) production. Furthermore, activation of P2X7 is accompanied by production of ROS, generated at least in part by NADPH oxidases [14,15]. In the context of redox regulation of target proteins, the cellular location and quantity of ROS generated seems to dictate the response. Overall, several studies using antioxidants support a model in which ROS production induced by Nlrp3 agonists drives inflammasome assembly [16].

The initial concept that NADPH oxidases are the primary source of ROS production during inflammasome activation [14] is becoming less accepted. Two independent studies utilizing mononuclear phagocytes from patients with chronic granulomatous disease demonstrated IL-1β secretion upon stimulation despite an inability of these cells to generate NADPH oxidase-dependent ROS because of a mutation of the $p47^{phox}$ subunit [17,18]. Because NADPH oxidase is not the only source of ROS in cells, the importance of mitochondrial-derived ROS has been recently explored. The mitochondrion is the main source of ROS under physiological conditions; however, under conditions of cellular stress, including increases in metabolic rates, hypoxia, or cellular disruption, the mitochondria can generate increased amounts of ROS [19]. In fact, blockage of key enzymes of the respiratory chain leads to ROS generation and consequent Nlrp3 inflammasome activation [20]. Despite several high-profile publications in the field, the mechanisms of production and the nature of ROS involvement in Nlrp3 inflammasome activation remain the subject of intense scrutiny.

Herein, we investigated the effects of mitochondria-derived ROS on Nlrp3 inflammasome activation in murine macrophages stimulated with serum amyloid A (SAA). Whereas SAA has been known as a biomarker of inflammation in several types of diseases [21], it can also stimulate cells via Toll-like receptor 2 (TLR2) and evoke a robust signaling cascade in human monocytes [22] and mouse macrophages [23]. More recently, we have demonstrated that SAA instillation into the lungs of mice elicits robust TLR2-, MyD88-, and IL-1-dependent pulmonary neutrophilic inflammation. We also demonstrated that in vitro SAA drives TLR2- and MyD88-dependent production of IL-1α, IL-1β, IL-6, IL-23, and prostaglandin E_2 by macrophages and dendritic cells. Furthermore, the production of IL-1β in response to SAA requires Nlrp3, ASC, and caspase-1 both in vitro and in vivo [24]. In this study, we report that SAA induces mitochondrial ROS and that uncoupling mitochondrial oxidative phosphorylation enhances Nlrp3 activation by preventing disassembly of the Nlrp3 complex.

Materials and methods

Chemicals and reagents

Ebselen; antimycin A; diphenyleneiodonium chloride (DPI); carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP); digitonin; adenosine 5′-triphosphate, periodate oxidized sodium salt (oxATP); apyrase, 4,4′,4″,4‴-(porphine-5,10,15,20-tetrayl)tetrakis (benzoic acid) (TBAP); protease inhibitor cocktail; and anti β-actin antibody were purchased from Sigma. Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), a superoxide dismutase (SOD) mimetic, was purchased from Calbiochem. The ROS detection reagent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), the mitochondrial ROS indicator MitoSOX red, and the mitochondrion-selective probe MitoTracker green were purchased from Molecular Probes/Invitrogen. The mitochondrial membrane potential indicator 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) was purchased from Cayman. The ATP bioluminescence assay kit (StayBrite) was purchased from BioVision. Ultrapure *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) and human recombinant SAA were purchased from InvivoGen and Peprotech, respectively. Nlrp3 and ASC antibodies were purchased from Enzo, antibodies for caspase-1 and IL-1β detection were purchased from Santa Cruz, and cytochrome *c* antibody clone 7H8.2C12 was purchased from BD Pharmingen. Secondary antibodies (anti-mouse–horseradish peroxidase (HRP) and anti-rabbit–HRP) were obtained from GE Healthcare. Dynabeads protein G for immunoprecipitation was purchased from Invitrogen. IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from BD Biosciences.

Cell culture and experimental conditions

Mice were housed in an American Association for the Accreditation of Laboratory Animal Care-approved facility at the University of Vermont, maintained on a 12-h light/dark cycle, and provided food and water ad libitum. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee. For the isolation of primary macrophages, C57BL/6 mice were administered 1 ml of 4% thioglycollate by ip injection. Ninety-six hours later, the mice were euthanized and peritoneal lavage was performed to collect peritoneal exudate cells. For isolation of human monocytes, we used freshly drawn peripheral blood of healthy donors and cultured the monocytes for 24 h before stimulation. Human monocytes, the mouse peritoneal macrophage cell line IC-21 (obtained from the ATCC), or primary mouse macrophages were seeded at 10^6 cells/ml using RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine and incubated at 37 °C in a 5% CO_2-supplemented atmosphere for at least 16 h before the appropriate treatments. SAA (1 μg/ml) or LPS (100 ng/ml) was utilized to stimulate primary human and mouse monocytes or IC-21 cells, respectively, at the indicated times. The antioxidants DPI (10 μM), ebselen (10 μM), and MnTBAP (100 μM) or its control TBAP (100 μM); the mitochondria-targeting drugs antimycin A (40 μg/ml) and FCCP (10 μM); apyrase (10 U/ml); and oxidized ATP (oxATP; 100 μM) were added before stimulation or to stimulated cells at the indicated times.

Immunoprecipitation and Western blot analysis

After the respective treatments, tissue culture plates were placed on ice and the attached cells were rinsed once with cold phosphate-buffered saline (PBS) and lysed using RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, and protease inhibitor cocktail (Sigma)). Lysates were removed from the plate, transferred to microcentrifuge tubes, and immediately frozen in liquid nitrogen to complete the lysis and prevent protein degradation. Alternatively, cell supernatants (medium) were concentrated by precipitation with 10% trichloroacetic acid followed by centrifugation at 1400 rpm for 20 min at room temperature (RT). The precipitates were neutralized and proteins resuspended in loading buffer [25]. Cell lysates or precipitates were equally loaded and proteins were separated by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred from the gel to a nitrocellulose membrane. The membrane was blocked for 1 h at RT with 3% milk and then incubated with the respective antibodies overnight at 4 °C on a rotating platform. The membranes were washed three times in PBS containing 0.05% Tween 20 and incubated with the respective HRP-conjugated secondary antibodies. Western blots were developed utilizing