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Original Contribution

MKK3 mediates inflammatory response through modulation of mitochondrial function

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ABSTRACT

Mitochondria are increasingly recognized as drivers of inflammatory responses. MAP kinase kinase 3 (MKK3), a dual-specificity protein kinase, is activated in inflammation and in turn activates p38 MAP kinase signaling. Here we show that MKK3 influences mitochondrial function and acts as a critical mediator of inflammation. MKK3-deficient ($MKK3^{-/-}$) mice and bone marrow-derived macrophages (BMDMs) secreted smaller amounts of cytokines than wild type (WT) after lipopolysaccharide (LPS) exposure. There was improved mitochondrial function, as measured by basal oxygen consumption rate, mitochondrial membrane potential, and ATP production, in $MKK3^{-/-}$ BMDMs. After LPS exposure, $MKK3^{-/-}$ BMDMs did not show a significant increase in cellular reactive oxygen species production or in mitochondrial superoxide compared to WT. Activation of two important inflammatory mediators, i.e., the nuclear translocation of NF-κB and caspase-1 activity (a key component of the inflammasome), was lower in $MKK3^{-/-}$ BMDMs. p38 and JNK activation was lower in $MKK3^{-/-}$ BMDMs compared to WT after exposure to LPS. Knockdown of MKK3 by siRNA in wild-type BMDMs improved mitochondrial membrane potential, reduced LPS-induced caspase-1 activation, and attenuated cytokine secretion. Our studies establish MKK3 as a regulator of mitochondrial function and inflammatory responses to LPS and suggest that MKK3 may be a therapeutic target in inflammatory disorders such as sepsis.

Mitochondria are considered central hubs of inflammation [1] and new paradigm shifts show that mitochondria are capable of inducing multiple inflammatory pathways, reflecting their origins as primitive archaebacteria [2]. Mitochondrial reactive oxygen species (ROS) have been implicated in the activation of major inflammatory transcription factors such as NF-κB (nuclear factor κB) and activator protein-1 [3]. In addition, mitochondrial ROS, membrane potential, and the release of oxidized mitochondrial DNA activate another important inflammatory pathway, the inflammasome and caspase-1 [4]. Mitochondrial metabolism and innate immunity are intimately linked. Metabolic switching from OXPHOS (oxidative phosphorylation) to glycolysis for ATP generation probably determines the magnitude of an inflammatory response. For example, OXPHOS deficiency is associated with increased inflammatory responses in adipocytes [5]. In macrophages and dendritic cells, stimulation of the Toll-like receptor 4 by lipopolysaccharide (LPS) inhibits mitochondrial activity and induces a change in the metabolism from oxidative phosphorylation to glycolysis, which is more inflammatory in nature [6,7]. Thus it is speculated that maintenance of adequate OXPHOS metabolism will enable reduced inflammatory response to stimulants such as LPS; however, the factors affecting mitochondrial function are not well known. Here we investigate the role of mitogen-activated protein (MAP) kinase kinase 3 (MKK3) in the regulation of inflammation through the modulation of mitochondrial function.

MAP kinases are integral to intracellular signaling pathways mediating inflammation, cell survival/death, proliferation, and differentiation in response to a wide variety of signals such as cytokines, growth factors, UV light, osmotic stress, and LPS [8]. MKK3 is a major upstream kinase of the p38 MAP kinase and phosphorylates p38 in response to LPS. MKK3 and p38 MAP kinase are ancient and evolutionarily conserved components of the metazoan defense against pathogen attack, which predated the canonical Toll-like-receptors [9]. We have previously described the role of MKK3 in sepsis, a syndrome of excessive and inappropriate inflammatory response to infection. We also found that MKK3 deficiency leads to less lung inflammation and better survival in various models of sepsis, such as cecal ligation and puncture and LPS exposure [10]. Additionally, we found that MKK3-deficient endothelial cells have a larger pool of healthy mitochondria as shown by higher mitophagy, clearance of dysfunctional mitochondria, and increased mitochondrial biogenesis [11]. It has been reported that mitochondrial ROS regulate MKK3 and p38, but MKK3 regulation of mitochondrial function has not been reported, to the best of our knowledge [12]. Thus, we hypothesized that MKK3 deficiency will have specific effects on mitochondria and this in turn will potently modulate inflammation.

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We sought to examine the effects of MKK3 deletion on mitochondrial function and cytokine secretion after LPS exposure in bone marrow-derived macrophages (BMDMs), which provide a good cell model to study inflammatory responses and mitochondrial function [13]. We show that MKK3 deletion leads to better mitochondrial function, lower production of ROS, and a reduction in NF-κB activation and caspase-1 activity and lower cytokine release after LPS stimulation. Thus, we conclude that mitochondrial dysfunction contributes to LPS-induced inflammatory responses in BMDMs and that MKK3 plays an important role.

Materials and methods

Animal experiments

The MKK3$^{-/-}$ mice were originally provided by R. Davis (University of Massachusetts Medical School, Worcester, MA, USA) and R. Flavell (Yale University, New Haven, CT, USA) and have been backcrossed onto a C57BL/6 background for >15 generations. We have previously described how the MKK3$^{-/-}$ mice were generated [10]. MKK3$^{-/-}$ mice expressed normal levels of MKK6, MKK4, JNK, and p38 MAP kinases [14]. The mice were divided into four groups of three each, i.e., wild-type control (C57BL/6 background), wild-type LPS treated, MKK3$^{-/-}$ control, and MKK3$^{-/-}$ LPS treated. LPS (*Escherichia coli* 055:B5; Sigma–Aldrich) was given at a single acute dose of 40 mg/kg ip (LD_{100}). Blood was collected after 3 h of LPS treatment for serum cytokine analysis. All experiments were conducted in accordance with the NIH guidelines and approved by the institutional animal care and use committee.

BMDM isolation and culture

BMDMs were isolated and differentiated using standard protocols. Primary macrophages were derived from bone marrow cells and were cultured for 7 days in RPMI 1640 medium containing 30% L929 cell conditioned medium, 10% fetal bovine serum, and penicillin/streptomycin (Pen/Strep). L929 cell conditioned medium was prepared by growing L929 cells in RPMI 1640 containing 10% fetal bovine serum and Pen/Strep for 10 days. BMDMs were harvested by treating with ice-cold Dulbecco's phosphate-buff ered saline (DPBS) containing 5 μM EDTA and plated as per experimental requirement.

Mitochondrial membrane potential (MMP)

BMDMs were seeded at a density 0.5×10^5 cells per well in a 96-well plate and exposed to LPS (0.1 μg/ml) after 16 h. Estimation of mitochondrial membrane potential ($\Delta\psi_m$) was performed using JC-10 (Enzo Life Sciences), a membrane-permeative fluorescent probe. JC-10 enters selectively into mitochondria and exists as two forms, monomeric or aggregate, depending upon the $\Delta\psi_m$. The JC-10 monomer form predominates in mitochondria with low $\Delta\psi_m$ and emits in the green wavelength (525–530 nm). The JC-10 aggregate form accumulates in mitochondria with high $\Delta\psi_m$ and emits in the orange wavelength (590 nm). The JC-10 aggregate/monomer ratio is proportional to the MMP. The final concentration of JC-10 used was 5 μM in the medium and it was incubated for 30 min before the fluorescence was read in a SpectraMax Gemini XS spectrofluorometer (Molecular Devices).

ATP measurement

Total ATP was determined using the ATP Fluorometric Assay Kit (BioVision). BMDMs, 0.5×10^6 cells per well in six-well cell culture plates, after 24 h LPS treatment were lysed in 100 μl of ATP assay buffer and centrifuged. The supernatant (50 μl) was mixed with the ATP probe, ATP converter, and developer mix and incubated for 30 min at room temperature in the dark. The fluorescence was read at ex 535/em 587 nm using a spectrofluorometer (VMax; Molecular Devices). The values were calculated against the provided standard.

Measurement of mitochondrial bioenergetics

An XF96 analyzer (Seahorse Biosciences, North Billerica, MA, USA) was used to measure bioenergetic function in intact BMDMs in real time. BMDMs were seeded into Seahorse Bioscience XF96 cell culture plates at the seeding density of 10,000 cells in 80 μl medium and allowed to adhere and grow for 24 h in a 37 °C humidified incubator with 5% CO_2. Measurements of extracellular flux were made in unbuffered medium. Extracellular acidification rate (ECAR) was calculated at baseline to compare the rate of glycolysis. Mitochondrial function was analyzed by sequentially adding pharmacological inhibitors of oxidative phosphorylation. The resultant bioenergetic profile provides detailed information on individual components of mitochondrial bioenergetic components.

Measurement of cellular ROS and mitochondrial superoxide in BMDMs

The cellular levels of ROS and mitochondrial superoxide were determined using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) and MitoSOX, respectively. BMDMs were cultured in 96-well plates at a density of 10,000 cells per well. After 16 h the medium was changed and CM-H_2DCFDA (final concentration 1 μM) and MitoSOX (final concentration 2 μM) were added along with nuclear stain Hoechst (5 μg/ml). After 60 min the cells were washed twice and suspended in DPBS and fluorescence was read at ex 352/em 461 nm for Hoechst, ex 492/em 527 nm for CM-H_2DCFDA, and ex 510/em 580 nm for MitoSOX. The cellular ROS and mitochondrial superoxide data were normalized to the nuclear stain and quantified.

Caspase-1 activity

BMDMs were seeded at 0.5×10^6 cells per well in six-well cell culture plates. After 24 h the medium was changed and LPS (0.1 μg/ml) treatment was done for 6 h with the addition of ATP (5 μM) for the last 15 min. Cells were lysed with cell lysis buffer provided in the Caspase-1 Fluorometric Assay Kit (ab39412; Abcam). Caspase-1 activity was determined using 100 μg cell lysate protein and the manufacturer's protocol. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate YVAD-*p*NA which absorbs at 405 nm. A standard curve for *p*NA was prepared and data are expressed as *p*NA released (μM)/mg protein.

NF-κB nuclear translocation immunostaining and image analysis

BMDMs were cultured on a coverslip in six-well cell culture plates at about 70% confluency. Cells were treated with LPS (0.1 μg/ml) for 45 min and cells were washed with DPBS twice and fixed in 4% paraformaldehyde for 15 min. After fixation the cells were washed with DPBS and permeabilized with 0.3% Triton X-100. After the cells were blocked with DAKO protein-free blocking buffer for 1 h, p-NF-κB (p65) antibody (Cat. No. 3031; Cell Signaling Technologies, Danvers, MA, USA) was added to the blocking buffer and incubated overnight. After two DPBS washes, anti-rabbit AF-488 (Cat. No. A11008; Invitrogen) secondary antibody was incubated for 1 h and washed with DPBS, and the cells were mounted with mounting medium containing