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Research paper

Fatty acid activates NLRP3 inflammasomes in mouse Kupffer cells through mitochondrial DNA release

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A B S T R A C T

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in many developed and developing countries worldwide. It has been well established that the chronic sterile inflammation caused by the NLRP3 inflammasome is closely related to NAFLD development. Kupffer cells (KCs) are involved in the pathogenesis of various liver diseases. We used methionine choline-deficient diets to establish a mouse nonalcoholic steatohepatitis (NASH) model. The expression and formation of the NLRP3 inflammasome in the KCs from the mouse and cell models were determined by Western blotting and co-immunoprecipitation. Evidence of mitochondrial DNA (mtDNA) release was determined by live cell labeling and imaging. KCs and the NLRP3 inflammasome exerted proinflammatory effects on the development and progression of NASH through secretion of the proinflammatory cytokine IL-1β. NLRP3, ASC and Caspase-1 protein expression levels in KCs from NASH mouse livers were significantly higher than those in KCs from NLRP3$^{-/-}$ mice, and the number of NLRP3 inflammasome protein complexes was significantly higher in KCs from NASH mouse livers, whereas these protein complexes could not be formed in NLRP3$^{-/-}$ mice. In in vitro experiments, palmitic acid (PA) decreased the mitochondrial membrane potential and subsequently induced mtDNA release from the mitochondria to the cytoplasm. NLRP3 inflammasome expression was substantially increased, and mtDNA-NLRP3 inflammasome complexes formed upon PA stimulation. Our data suggest that mtDNA released from mitochondria during PA stimulation causes NLRP3 inflammasome activation, providing a missing link between NLRP3 inflammasome activation and NASH development, via binding of cytosolic mtDNA to the NLRP3 inflammasome.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is currently the most common cause of chronic liver disease in many developed and developing countries in the world [1]. NAFLD is defined as steatosis affecting more than 5% of hepatocytes in the absence of excessive alcohol consumption, the consumption of steatogenic drugs or other liver disease [2]. The histological spectrum of NAFLD includes nonalcoholic fatty liver (NAFL), steatohepatitis (NASH), fibrosis, cirrhosis, possibly progressing to hepatocellular carcinoma after 10–20 years [3].

Day and James proposed the 'double-hit' hypothesis in 1998, aiming to describe the pathogenesis of NAFLD [4]. Recently, it was acknowledged that the step from NAFL to NASH is essential during the development of NAFLD [5]. Metaflammation, a low-grade form of chronic sterile inflammation, is an essential event in the development of NASH [6]. Furthermore, epidemiological evidence suggests a close relationship between NAFLD and unhealthy lifestyles, and diet and lifestyle changes are recommended for NAFLD patients. However, no specific therapy can be firmly recommended [7].

The nucleotide binding domain and leucine-rich repeat pyrin 3

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; NAFL, Non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; FFA, free fatty acid; PA, palmitic acid; KCs, Kupffer cells; MCD, methionine choline deficient; WT, wild type; Co-IP, co-immunoprecipitation; LC-MS, liquid chromatography-mass spectrometry; DAMPs, danger-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; CypD, cyclophilin D; TSPO, translocator protein

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domain (NLRP3) scaffold protein orchestrates the formation of the best-characterized inflammasome to date, consisting of the NOD-like receptor NLRP3, the adaptor ASC and the effector pro-caspase-1 [8]. The NLRP3 inflammasome is triggered by several danger signals including toxins from bacteria and danger signals released by damaged or stressed cells. The precise molecular mechanisms by which these different stimuli signal the assembly and activation of the NLRP3 inflammasome remain largely unknown, and none of these stimuli have been found to directly bind and activate NLRP3 [9].

Mitochondria sense cellular danger that results in apoptosis, during which mtDNA is released into the cytoplasm and binds to NLRP3, and this cascade of events results in activation of the NLRP3 inflammasome and caspase-1 maturation [10]. Our previous study found that NLRP3 is a proinflammatory factor in the progression of NASH, and free fatty acid (FFA) acts as a kind of damage-associated molecular pattern (DAMP) to activate the NLRP3 inflammasome in a NASH mouse model, during which the majority of NLRP3 inflammasomes is expressed in the mitochondrial fraction [11]. Nevertheless, the molecular mechanisms by which FFA activate the NLRP3 inflammasome in the NASH mouse model remains unclear.

Kupffer cells (KCs) comprise liver-resident populations of macrophages that are involved in the pathogenesis of various liver diseases such as steatohepatitis, viral hepatitis and nonalcoholic liver disease [12–14]. Our previous study showed that NLRP3 inflammasome activation in KCs exerted harmful effects in the progression of NASH [11], and the KCs were the dominant source of the proinflammatory cytokine IL-1β compared with other cell types in the liver such as hepatocytes and hepatic stellate cells (HSCs) [15,16].

In the present study, we explored the roles of NLRP3, ASC and caspase-1 in NASH development and the exact mechanisms of NLRP3 inflammasome activation in KCs induced by FFA. We hypothesized that NLRP3 knockout would prevent the development of mouse NASH and that FFA stimulation would lead to mtDNA release into the cytoplasm, binding to NLRP3 and inducing IL-1β secretion through activation of the NLRP3 inflammasome in KCs.

2. Materials and methods

2.1. Animals and diets

NLRP3 knockout ($NLRP3^{-/-}$) male mice were purchased from Jackson Laboratories (Bar Harbor, ME) were bred in the laboratory animal research center of Chongqing Medical University (Chongqing, China). Wild-type (WT) C57BL/6 mice were obtained from the laboratory animal research center of Chongqing Medical University. We divided the WT and $NLRP3^{-/-}$ mice (8 weeks, 10 mice in each group) into two groups: normal diet (ND) or methionine choline deficient (MCD) diet (A02082002B, Research Diets, USA) for 5 weeks. The animals received humane care in compliance with the institution's guidelines, as outlined in the guide for the care and use of laboratory animals prepared by the National Academy of Sciences. All mice were housed under specific pathogen-free condition and allowed free access to sterile water and food.

2.2. Histological analysis

Sections of formalin-fixed livers were underwent hematoxylin-eosin (HE), Oil Red O, and immunohistochemical staining for NLRP3 (ab4207, Abcam, UK), ASC (sc-22514-R, Santa Cruz, USA), and Caspase-1 (sc-56036, Santa Cruz, USA). The NASH activity score was evaluated by two pathologists. The quantitative immunohistochemical staining values (QISVs) were calculated as the integrated OD, which was divided by the total area occupied by the brown cells.

2.3. Serum analysis

Serum aminotransferase levels were measured by rate method. Serum FFA levels were determined using an enzymatic method (Clinimate NEFA kit, Sekisui Medical Company, Japan).

2.4. In vitro experiments

Primary KCs were isolated from mouse livers according to a previously method [17]. Briefly, animals were anaesthetized by diethyl ether inhalation. The liver was perfused in situ with 10 ml PBS at 37 °C through portal vein. The liver was then excised, transferred to a 60 mm culture dish and the tissue was minced to small pieces. The liver tissues were dispersed in 10 ml Roswell Park Memorial Institute 1640 (RPMI 1640, Hyclone) containing 0.1% type IV collagenase and bathe-watered at 37 °C for 30 min, mixed gently with graduated pipette up and down per 10 min. Following digestion, the liver homogenate was filtered through a 74 μm stainless steel wire mesh to remove undigested tissue and the cell suspension was centrifuged at 300 × *g* for 5 min at 4 °C. The cell sediment was reserved. KCs were further separated from hepatocytes and other sinusoidal cells by gradient centrifugation. KCs from WT and $NLRP3^{-/-}$ mice (8 weeks, 10 mice in each group) were randomly divided into two groups: the control (CON) groups and palmitic acid stimulated (PA, 0.32 mM) groups. Cell culture supernatant (SN) and NLRP3 inflammasome protein expressions were further analysed.

2.5. Western blotting analysis

The protein expression levels of NLRP3, ASC and Caspase-1 in KCs were detected by western blotting. Briefly, we obtained protein extracts through homogenizing samples in lysis buffer (R0278, Sigma, UK). Protein concentration was determined by BCA protein assay method (23227, Thermo, UK). Equal amount of protein samples was separated by electrophoresis, and then transferred onto a polyvinylidene fluoride membrane. We blocked the membrane for 1 h with 5% non-fat dry milk, and then incubated with primary antibodies (NLRP3, ab4207, Abcam, UK, ASC, sc-22514-R, Santa Cruz, USA, and Caspase-1, sc-56036, Santa Cruz, USA) at 4 °C overnight. On the second day, we washed the membrane and incubated for 1 h at 37 °C with the secondary antibodies. Finally, the membrane was exposed to an auto radiographic film (Kodak, USA). The relative amount of the proteins was quantified by relative density of protein bands using the image analysis system. For the protein expression of IL-1β in culture supernatant, we firstly used the Amicon® Ultra-4 10 K centrifugal filter devices (Merck Millipore) to concentrate the culture supernatant, and then assessed the protein expressions of pro-IL-1β and IL-1β by western blot (ab9722, Abcam, UK).

2.6. Co-immunoprecipitation

The interactions between mtDNA and NLRP3 inflammasome proteins in KCs were analysed by co-immunoprecipitation (Co-IP). Briefly, KCs were preloaded with BrdU (10 μM, ab142567, Abcam) for 24 h at 37 °C in a CO_2 incubator and treated as indicated. The remaining steps are the same as our previous method [11].

2.7. Measurement of mitochondrial membrane potential and mtDNA release

Cells were stained with the TMRM (Tetramethylrhodamine, T668, Invitrogen) as described in the manufacturer's protocol. Cells were loaded with 200 nM of TMRM for 30 min, and then washed three times with PBS. TMRM fluorescence was measured by fluorescence microscopy (ZEISS LSM 710, Germany). Cells were stained with the picogreen (P11495, Invitrogen) and mitotracker red (M7512, Invitrogen). Laser power was kept as low as possible to avoid bleaching of the signal. The Mander's overlap coefficient values were analysed for the degree of co-localization of mtDNA and mitochondria. Images were analysed using