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Palmitic acid elicits hepatic stellate cell activation through inflammasomes and hedgehog signaling

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ABSTRACT

Aims: Activation of hepatic stellate cells (HSCs) plays a pivotal role at the center of the fibrogenic progression in nonalcoholic steatohepatitis (NASH). However, it is poorly understood that how various molecules interact within HSCs during the progression of NASH to fibrosis. The aim of the present study is to delineate how inflammasome molecules, hedgehog signaling and autophagy provoke HSC activation using palmitic acid (PA) as a major insult.

Main methods: Inflammasome activation, hedgehog signaling activity and autophagy in PA-exposed HSCs were determined to investigate their role in activation of human and rodent HSC lines or primary HSCs.

Key findings: PA treatment elicited HSC activation reflected by increased mRNA levels of transforming growth factor-β1, connective tissue growth factor, tissue inhibitor of metalloproteinase-1 and procollagen type I (α1). In addition, expression levels of NOD-like receptor protein 3 (NLRP3) and hedgehog signaling transcription factor Gli-1 were increased in PA-exposed HSCs. It's evident that PA treatment resulted in increased production of light chain 3-II and autophagosomes, as well as enhanced autophagy flux reflected by transduction of an adeno-associated viral vector. Whereas, reduced autophagy, which is often seen in the late stage of NASH, provoked inflammasome activation. Moreover, suppressing the Hh signaling pathway by LDE225 blocked production of light chain 3-II and autophagy flux.

Significance: Saturated fatty acids, such as PA, stimulate HSC activation through inflammasomes and hedgehog signaling. Meanwhile, compromised autophagy may facilitate HSC activation, implicating valuable candidates for pharmacologic intervention against the progression of fibrogenesis in NASH.

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1. Introduction

Twenty to 25% individuals with simple fatty liver will progress to nonalcoholic steatohepatitis (NASH) in 5–10 years [1]. Excessive accumulation of triglyceride (TG) in hepatocytes is the hallmark of NASH, and increased free fatty acid content in hepatocytes accounts for hepatocellular injury and death through oxidant stress, endoplasmic reticulum (ER) stress, insulin resistance and apoptosis or pyroptosis [2]. It has been known that hepatocellular injury and death are the initial and sustained stimuli for fibrogenesis, in which activation of hepatic stellate cells (HSCs) with a transition from a quiescent phenotype to myofibroblast-like cells is the center of the complex paradigm [3]. However, how HSCs are activated in a steatotic microenvironment, especially with increased fatty acid influx into the liver, is poorly understood. Therefore, in the present study, our intent is to investigate how fatty acids activate HSCs and the consequences using primary HSCs, human and rodent immortalized HSC lines as a platform for an initial pharmacological evaluation.

Inflammasome molecules are multiprotein complexes formed by NOD-like receptor (NLR) family members, and represent a family of recognition receptors which identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in a variety of cell types [4]. Inflammasome activation has been recently recognized to play a central role in the development of carbon tetrachloride-induced and obesity-associated liver injury and fibrosis [5,6]. Among NLRs, NLRP3 senses a wide array of stimuli from the extracellular space or subcellular compartments, and participates in the process of innate immune defense. It cleaves and activates pre-caspase-1 to caspase-1, which in turn activates IL-1β and IL-18, resulting in pyroptosis [7]. However, whether inflammasomes directly affect HSC activation in a steatotic microenvironment is unknown.

The hedgehog (Hh) signaling pathway is a highly conserved cross species, and orchestrates multiple aspects of embryogenesis, development and oncogenesis [8]. Enhanced activation of the Hh signaling pathway has been shown in patients with alcoholic, non-alcoholic steatohepatitis and primary biliary cholangitis [9,10], and thought to

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be a key factor for cross-talk between hepatocytes and HSCs during hepatic injury and fibrosis [11,12]. However, how Hh signaling modulates HSC activation in steatohepatitis is poorly understood.

Autophagy is an intracellular pathway that breaks down damaged organelles and long-lived proteins in lysosomes to promote cell survival under various conditions, such as starvation or lipid overload [13]. It has been shown recently that autophagy mediates the breakdown of lipid droplets in hepatocytes and therefore participates in the development of hepatic steatosis [14]. Not surprisingly, intracellular droplets disappear during the process of HSC activation, which has been found to be the results of autophagy [15]. Therefore, how free fatty acids activate inflammasomes and Hh signaling in concurrence of autophagy is a crucial question in revealing the molecular basis of hepatic fibrogenesis during steatohepatitis. In this present study, we evaluate the hypothesis that activated inflammasomes and up-regulated Hh signaling participate in the activation of palmitic acid (PA)-exposed HSCs, and that autophagy interacts with these two signaling events contributing to the perplex of HSC activation.

2. Materials and methods

2.1. Sources of materials

Palmitic acid (PA, Sigma-Aldrich, St. Louis, MO), dissolving in methanol (stock solution 31.2 mM), was mixed with 5% fatty acid-free bovine serum albumin (BSA) in phosphate buffered saline (PBS) at a molar ratio of 5:1, and the final concentration was 200 μM. *S*-Adenosyl-L-methionine (SAMe), parthenolide and chloroquine (CQ) (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water, and pH of the stock solution was adjusted to 7.4. LDE225 (Cellagen Technology, CA, USA), a potent inhibitor of Smo, which is a critical transmembrane molecule in the Hh signaling pathway [16], was dissolved in dimethyl sulfoxide (DMSO) and diluted in medium. All stock solutions were kept at −20 °C till the use in experiments.

2.2. Cell culture and treatment

Three immortalized stellate cell lines were used in this study: human LX2 [17], human immortalized HSC [9] and rat BSC-C10 cells [18,19], which are partially-activated HSCs obtained separately from Drs. Scott L. Freidman, Mt. Sinai School of Medicine, New York, NY; David Brenner, University of California School of Medicine, San Diego, CA, and Hidekazu Tsukamoto, Keck School of Medicine of University of Southern California, Los Angeles, CA. Cells were cultured in DMEM with 10% fetal bovine serum (FBS, Gibco Life Technologies, Grand Island, NY), 1% (v/v) sodium pyruvate, 1% (v/v) glutamine and 1% (v/v) penicillin-streptomycin at 37 °C in 5% CO_2 and 95% air-humidified incubator. HSCs at 1×10^5 were seeded in 6-well plates for 24 h, then exposed to PA at 200 μM with or without pretreatment with SAMe (1 mM), parthenolide (5 μM), LDE225 (10 nM) or CQ (25 μM).

2.3. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from HSCs exposed to PA with or without the preincubation with SAMe, parthenolide, LDE225 or CQ by Trizol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocols. RNA was converted to cDNA by reverse transcription using PrimeScript™ RT Reagent Kit (Takara Bio Inc., Dalian, China). qRT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Relative gene expression was normalized to the housekeeping gene, human or rat glyceraldehyde phosphate dehydrogenase (GAPDH), and expressed as $2^{(-\Delta\Delta CT)}$ as previously described [20]. All primers were synthesized by Shenggong Biotech (Shanghai, China), and their sequences are shown in Supplemental Table 1.

2.4. Western blot analysis

Total protein was extracted with RIPA lysis buffer (Ruian BioTechnologies, Shanghai, China) and centrifuged at 12,000*g* for 15 min at 4 °C. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, USA). Fifty microgram of protein was subjected to sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). After being blocked with 5% BSA at room temperature (RT) for 2 h, the membranes were immunoblotted with the primary antibodies (1:500 dilutions) at 4 °C overnight, and further with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilutions). Blots were imaged using an ECL detection system (Tanon, Shanghai, China). GAPDH was used as an equal protein loading control as we reported previously [21]. All antibodies used in the experiments are shown in Supplemental Table 2.

2.5. Isolation of rat hepatic stellate cells

Animal experimental protocol was approved by the Animal Ethnic Committee of Fudan University School of Basic Medical Sciences, and all procedures follow the NIH guidelines of experimental animal handling and care. Primary HSCs were isolated from male Sprague-Dawley rats weighing about 500 g. The method of isolating primary HSC was reported previously by us [22,23]. In brief, rats were anesthetized with sodium pentobarbital intraperitoneally (60 mg/kg). After being perfused with Ca^{2+}, Mg^{2+}-free solution, pronase E and collagenase type IV (Sigma) solution, parenchymal cells were digested with DNase treatment. Finally, HSCs were separated by Nycodenz® gradient centrifugation, and cultured with M199 medium (Hyclone, Shanghai, China) containing 20% FBS and 1% penicillin-streptomycin for first 48 h, and then in M199 medium containing 10% FBS overtime.

2.6. Staining of lysosomes in HSCs

Lysotracker Red (Beyotime Biotechnology, Shanghai, China) was dissolved in M199 medium and kept at −20 °C. Primary rat HSCs were grown on coverslips (Winhongbio, Shanghai, China) for 6 days and then cultured with PA at 200 μM for 3 h. Lysotracker-red stock solution (1 μM) was added to M199 medium containing PA (37 °C) to reach a final concentration at 100 nM and co-cultured with cells for 1 h. After washing with PBS for 3 times, cells were examined under a fluorescent microscope.

2.7. Immunofluorescent staining

After treatment, HSCs on coverslips were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. Cells were blocked with 5% BSA for 1 h, and then incubated with primary anti-smooth muscle α-actin (α-SMA) or anti-LC3 antibody (1:50 in 1% BSA) at 4 °C overnight. After washing, cells were incubated with secondary fluorescein isothiocyanate (FITC)-conjugated antibody (1:50 in 1% BSA) for 1 h at 37 °C. Then cells were incubated with 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min [24]. Finally, cells were examined under a confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.8. mRFP-GFP-LC3 adeno-associated viral transduction for detection of autophagy flux

As autophagy is a dynamic process, the detection of LC3 processing by Western blot and visualization of autophagosome formation by fluorescent staining are insufficient to reflect autophagic activity. Therefore, we infected cells with an adeno-associated viral vector (AAV-mRFP-GFP-LC3, HanBio Technology Co. Shanghai, China), which dynamically

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