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Protective role of autophagy in methionine–choline deficient diet-induced advanced nonalcoholic steatohepatitis in mice ☆

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ABSTRACT

The methionine choline-deficient (MCD) diet leads to severe liver injury similar to human nonalcoholic steatohepatitis (NASH). Autophagy has emerged as a critical lysosomal pathway that maintains cell function and survival through the degradation of cellular components such as organelles and proteins. The goal of this study was to elucidate the role of autophagy in MCD-induced steatosis, fibrosis, inflammation, mitochondrial dysfunction, and endoplasmic reticulum (ER) stress in mice. Mice were fed with MCD diet and treated with rapamycin (an autophagy enhancer) or chloroquine (an autophagy inhibitor) for 10 weeks. Liver injury was evaluated biochemically and histologically together with hepatic gene expression analysis. Autophagic flux was impaired in livers of mice fed with MCD diet, evidenced by reduced ratio of LC3-II/LC3-I and increased protein expression of p62. It was found that autophagy activation by rapamycin attenuated MCD-induced steatosis, fibrosis, inflammation, mitochondrial dysfunction, and ER stress. By contrast, MCD mice treated with chloroquine developed more liver injury. In conclusions, the autophagic pathway plays an important protective role in MCD-induced advanced NASH. Thus, pharmacological promotion of autophagy may provide a novel therapeutic strategy for treatment of NASH.

1. Introduction

Nonalcoholic steatohepatitis (NASH), as a progressive disease, is a significant predisposing factor for the development of cryptogenic cirrhosis, hepatic failure, and hepatocellular carcinoma (Cohen et al., 2011). Twenty percent of NASH patients are reported to develop cirrhosis, and 30–40% of individuals with NASH cirrhosis experience liver-related death (McCullough, 2006). Despite its clinical significance, there is a lack of effective and appropriate pharmacotherapy for NASH, currently. An increasing understanding of its pathogenesis will likely improve the development of treatment and interventions in the future.

Autophagy is a genetically programmed, evolutionarily-conserved catabolic process, which is manifested as degradation of cellular proteins and damaged organelles in an effort to promote cell survival and maintain cellular homeostasis. In Huh7 hepatic cells, short-time treatment with palmitic acid triggered activation of the autophagic flux. Conversely, prolonged treatment with palmitic acid induced a blockade of the autophagic flux (González-Rodríguez et al., 2014; Mei et al., 2011). Impaired autophagy flux was found in livers of NASH patients and mice fed with high-fat diet (HFD) and obese (*ob*/*ob*) mice (González-Rodríguez et al., 2014; Mei et al., 2011; Zeng et al., 2015). Inhibition of autophagy in cultured hepatocytes and mouse liver increased triglyceride storage in lipid droplets (Singh et al., 2009). Furthermore, HFD-fed mice with a hepatocyte-specific knockout of Atg7 developed markedly accumulation of liver triglyceride and cholesterol; and adenoviral-mediated Atg7 overexpression attenuated NASH in obese (ob/ob) mice (Yang et al., 2010; Singh et al., 2009). Autophagy deficiency by hepatic FIP200 deletion aggravated steatosis from liver injury in HFD-fed mice (Ma et al., 2013).

Abbreviations: ALT, alanine aminotransferase; CHOP, CCAAT enhancer binding protein homologous protein; CPT-I, carnitine palmitoyl transferase-I; CQ, chloroquine; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; LC3, microtubule-associated protein 2 light chain 3; MCD, methionine-choline-deficient; MCP-1, monocyte chemotactic protein 1; MPO, myeloperoxidase; NASH, nonalcoholic steatohepatitis; PGC-1α, α subunit of peroxisome proliferators-activated receptor-γ coactivator-1; PPARα, peroxisome proliferator-activated receptor α; RP, rapamycin; TFAM, transcription factor A of mitochondria; XBP-1, X-box protein 1; α-SMA, alpha smooth muscle actin

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Pharmacological promotion of autophagy by carbamazepine or rapamycin alleviated steatosis and injury in HFD-fed mice (Lin et al., 2013). However, standard HFD generally do not induce significant NASH (i.e., liver cell death, inflammation, or fibrosis) even when fed for more than 28 weeks, despite reproducibly provoking obesity, the metabolic syndrome, and hepatic steatosis (Asai et al., 2014). Despite the presence of profound fatty liver, obesity, and diabetes, obese (*ob/ob*) mice do not develop significant liver injury because of the essential role of leptin in hepatic injury and fibrosis (Leclercq et al., 2002; Aleffi et al., 2005). MCD diet is a very reproducible model, consistently inducing a phenotype of severe NASH after 8 weeks of administration (Itagaki et al., 2013). More importantly, When compared to MCD diet model, various mechanisms implicated in human NASH pathogenesis/progression were also less robust in the HFD diet model, including oxidative stress, ER stress, autophagy deregulation, and hedgehog pathway activation (Machado et al., 2015).

To deeply define the role of autophagy in NASH, MCD diet model was applied in this work, and we thus investigated whether pharmacological activation or inhibition of autophagy modulated MCD-induced severe NASH in mice and explored its underlying mechanism associated with mitochondrial dysfunction, inflammation, and ER stress.

2. Materials and methods

2.1. Animals and study design

C57BL/6J mice (6-week-old) were obtained from the Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). All the animals were entrained to controlled temperature (23 ± 2 °C) with 12:12-h light:dark cycles and free access to water and diet. The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Second Military Medical University.

All the mice were randomly divided into four groups as follows: (1) mice fed with control diet; (2) mice fed with Methionine and Choline Deficient Diet (MCD, Composition of diet was shown in Supplementary data, Table S2); (3) mice fed with MCD diet and treated with rapamycin (an autophagy enhancer, Sigma, 0.25 mg/kg/day, intraperitoneally) (Das et al., 2014); (4) mice fed with MCD diet and treated with chloroquine (an autophagy inhibitor, Sigma, 20 mg/kg/day, intraperitoneally) (Xiu et al., 2014). Ten weeks later, mice were anesthetized by intraperitoneally administration of 50 mg/kg pentobarbital, and serum and livers were collected.

2.2. Western blotting analysis

Twenty micrograms total protein were separated on 10% gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinyldifluoride membranes (GE Healthcare, Buckinghamshire, UK). Antibodies against LC3 (1:1000; Abcam, #ab48394) and p62 (1:1000; Cell Signaling Technology, #5114) were used for western blotting analysis. Bands were visualized with the enhanced chemiluminescence reagent and digitized using a CCD camera (ChemiDoc®, Bio-Rad, Hercules, CA, USA). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:8000; Sigma-Aldrich, #G9545) to correct for variations in sample loading and are expressed as percentages of control signals (% control) in each blot to correct for variations between blots.

2.3. Liver histology

Liver tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Slides were blindly evaluated and scored for steatosis, ballooning, and inflammation. Steatosis (0–4): 0 = < 5%; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%. Inflammation (0–4): 0 = absent; 1 = minimal (0–1 focus per 20 × field); 2 = mild (two foci); 3 = moderate (three foci); 4 = severe (four or more foci). Ballooning (0–3): 0 = absent; 1 = mild (focal involving fewer than three hepatocytes); 2 = moderate (focal involving more than three

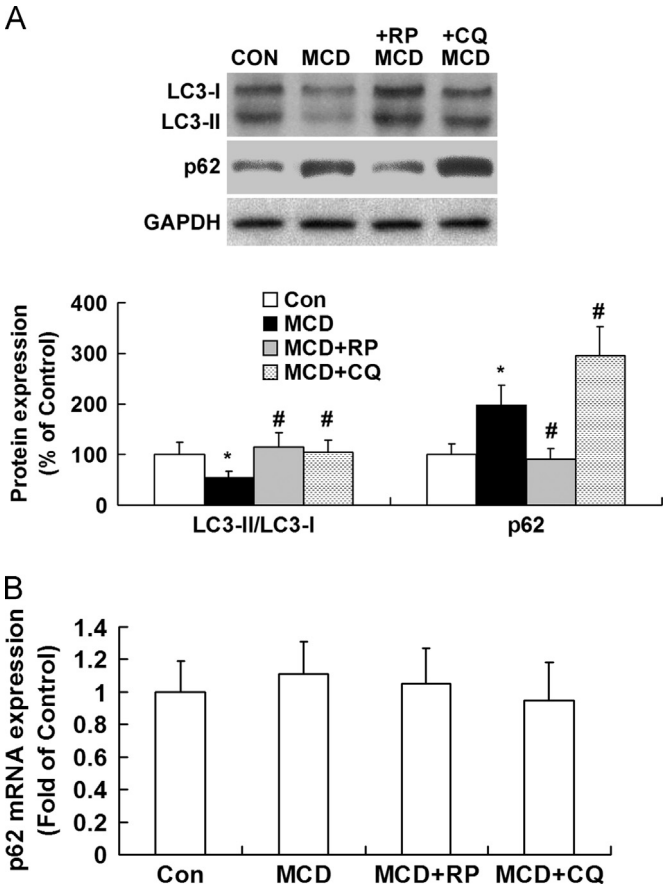

Fig. 1. Autophagy in livers of mice fed with MCD diet. Mice were fed with MCD diet and treated with rapamycin or chloroquine for 10 weeks. Representative Western blotting results and quantification analysis (A) of LC3-II, LC3-I, and p62 were showed. Graphs showed the mRNA levels of p62 (B) in livers. Values are means ± S.D. n = 9–11 in each group; MCD, methionine-choline-deficient; RP, rapamycin; CQ, chloroquine; LC3, microtubule-associated protein 2 light chain 3; *$P < 0.05$ versus control group; #$P < 0.05$ versus MCD group.

Table 1
Biochemical parameters in mice.

	Con	MCD	MCD+RP	MCD+CQ
Liver/body weight (%)	4.3 ± 0.3	5.8 ± 0.4*	4.8 ± 0.5**	6.6 ± 0.5**
Serum				
Glucose (mg/dl)	301.3 ± 52.6	146.9 ± 43.7*	155.5 ± 47.2*	141.2 ± 40.1*
Total cholesterol (mg/dl)	170.5 ± 36.9	76.9 ± 24.4*	82.7 ± 18.7*	73.8 ± 22.7*
Triglycerides (mg/dl)	142.6 ± 30.2	72.5 ± 17.8*	75.9 ± 20.4*	68.5 ± 16.6*
Liver				
Triglycerides (mg/g liver)	26.9 ± 6.8	90.7 ± 13.4*	55.3 ± 10.7**	119 ± 17.5**

Values are means ± S.D. n = 9–11 in each group; MCD, methionine-choline-deficient; RP, rapamycin; CQ, chloroquine.

* $P < 0.05$ versus control group.

** $P < 0.05$ versus MCD group.