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Research paper

Caveolin 1-related autophagy initiated by aldosterone-induced oxidation promotes liver sinusoidal endothelial cells defenestration

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ABSTRACT

Aldosterone, with pro-oxidation and pro-autophagy capabilities, plays a key role in liver fibrosis. However, the mechanisms underlying aldosterone-promoted liver sinusoidal endothelial cells (LSECs) defenestration remain unknown. Caveolin 1 (Cav1) displays close links with autophagy and fenestration. Hence, we aim to investigate the role of Cav1-related autophagy in LSECs defenestration. We found the increase of aldosterone/MR (mineralocorticoid receptor) level, oxidation, autophagy, and defenestration in LSECs in the human fibrotic liver, BDL or hyperaldosteronism models; while antagonizing aldosterone or inhibiting autophagy relieved LSECs defenestration in BDL-induced fibrosis or hyperaldosteronism models. *In vitro*, fenestrae of primary LSECs gradually shrank, along with the down-regulation of the NO-dependent pathway and the augment of the AMPK-dependent autophagy; these effects were aggravated by rapamycin (an autophagy activator) or aldosterone treatment. Additionally, aldosterone increased oxidation mediated by Cav1, reduced ATP generation, and subsequently induced the AMPK-dependent autophagy, leading to the down-regulation of the NO-dependent pathway and LSECs defenestration. These effects were reversed by MR antagonist spironolactone, antioxidants or autophagy inhibitors. Besides, aldosterone enhanced the co-immunoprecipitation of Cav1 with p62 and ubiquitin, and induced Cav1 co-immunofluorescence staining with LC3, ubiquitin, and F-actin in the perinuclear area of LSECs. Furthermore, aldosterone treatment increased the membrane protein level of Cav1, whereas decrease the cytoplasmic protein level of Cav1, indicating that aldosterone induced Cav1-related selective autophagy and F-actin remodeling to promote defenestration. Consequently, Cav1-related selective autophagy initiated by aldosterone-induced oxidation promotes LSECs defenestration via activating the AMPK-ULK1 pathway and inhibiting the NO-dependent pathway.

1. Introduction

Autophagy, a constitutive process that mediates encapsulation of damaged proteins, lipids or organelles in double-membrane autophagosomes for degradation, contributes to the maintenance of cellular homeostasis [1]. As a result, moderate autophagy facilitates cellular

Abbreviations: 3MA, 3-methyladenine; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; ATP1B2, ATPase Na+/K+ transporting subunit beta 2; BDL, bile duct ligation; Cav1, Caveolin 1; CD31, platelet endothelial cell adhesion molecule-1, PECAM-1; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; LC3, microtubule-associated protein 1 light chain 3; LSEC, liver sinusoidal endothelial cell; MR, mineralocorticoid receptor; NAC, N-acetyl-L-cysteine; NO, nitric oxide; PKG, protein kinase G; ROS, reactive oxygen species; SEM, scanning electron microscopy; sGC, soluble guanylatecyclase; TEMPO, 2,2,6,6-tetramethylpiperidinooxy; mito-TEMPO, mitochondria 2,2,6,6-tetramethylpiperidinooxy; TEM, transmission electron microscopy; ULK1, unc-51 like autophagy activating kinase 1; VASP, vasodilator-stimulated phosphoprotein; vWF, von Willebrand Factor

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recycling and survival against extra-cellular detrimental stimuli, such as low nutrients, low ATP, and reactive oxygen species (ROS) [2]. Autophagy plays an essential role in the regulation of intra-hepatic cell function and metabolism. Autophagy attenuates the abnormal protein aggregates and protects hepatocytes against injury [3]. However, for hepatic stellate cells (HSCs), autophagy degrades triglyceride to activate HSCs [4].

In pathological conditions, liver sinusoidal endothelial cells (LSECs) display defenestration and capillarization, which initiates liver fibrosis. Recently, autophagy is reported to modulate the phenotype of LSECs and protect against acute liver injury induced by ischemia/reperfusion (I/R) [5]. However, how autophagy impacts on LSECs defenestration and capillarization, the preliminary step necessary for fibrogenesis and portal hypertension, is still unknown.

Aldosterone, the effector of the renin-angiotensin-aldosterone system (RAAS), has been evidenced to play a key role in organ fibrosis, such as in heart, renal, and liver tissue, via its pro-oxidative capacity [6,7]. Interestingly, aldosterone induced podocyte autophagy via oxidation [8], and the ROS-induced autophagy feed-back opposed aldosterone-induced podocyte injury by attenuating endoplasmic reticulum stress [9]. Given the pro-oxidation and pro-autophagy capabilities of aldosterone, in the present study, we focus on the effects of aldosterone on defenestration of LSECs and capillarization via initiation of oxidation and autophagy, which precedes the activation of HSCs and liver fibrogenesis. The mechanism of aldosterone in this process remains unclear.

Caveolin 1 (Cav1), a scaffolding/regulatory protein of caveolae on the plasma membrane of caveolae and on vesicles, displays close links with oxidation and autophagy. As a lipid raft molecule of caveolae in the membrane, Cav1 interacts with mineralocorticoid receptors (MR) and forms an MR/Cav1 complex in caveolae, which mediates a rapid signaling cascade of oxidation initiated by aldosterone [10]. The Cav1-mediated NADPH oxidase (NOX) promoted autophagy of intestinal epithelial cells [11]. In addition, Cav1 could interact with ATG12-ATG5 system to suppress autophagy in lung epithelial cells [12].

Moreover, being part of the cytoskeleton around fenestrae, F-actin modulates contraction of fenestrae [13], whose remodeling may facilitate defenestration. Autophagosome maturation was promoted by F-actin remodeling, and then, in turn, assembled an F-actin network and facilitated remodeling [14], indicating that autophagy contributes to F-actin remodeling and subsequent fenestrae contraction.

Consequently, Cav1 may be a multifunctional signaling hub that allows it to regulate aldosterone-induced oxidation, autophagy, and LSECs defenestration. Hence, the present study aims to investigate the role of Cav1-related autophagy in LSECs defenestration. We demonstrated for the first time that Cav1-related autophagy initiated by aldosterone-induced oxidation promotes LSECs defenestration.

2. Materials and methods

2.1. Reagents and antibodies

The reagents used included aldosterone (Sigma-Aldrich A9477, 52391), spironolactone (Sigma-Aldrich, S4054), 3MA (Sigma-Aldrich, S2767), rapamycin (Sigma-Aldrich, S1039), bafilomycin A1 (Sigma-Aldrich, SML1661), N-acetyl-L-cysteine (NAC, Sigma-Aldrich, A9165), TEMPO (Sigma-Aldrich, 426369), mito-TEMPO (Sigma-Aldrich, SML0737).

The antibodies used included anti-α-SMA (Boster, BM0002), anti-vWF (Santa Cruz, SC-365712), anti-vWF (Abcam, ab174290), anti-CD31 (Santa Cruz, SC-46694), anti-Cav1 (Abcam, ab17052), anti-Cav1 (Abcam, ab2910), anti-MR (Abcam, ab2774), anti-LC3 (Abcam, ab48394), anti-CD32b (Abclonal, A7554), anti-ubiquitin (Abcam, ab19247), anti-NOX4 (Abcam, ab60940), anti-p62 (Abcam, ab155686), anti-VASP (CST, 3132S), anti-eNOS (Abclonal, A1548), anti-AMPK (Proteintech, 10929-2-AP), anti-p-AMPK(Thr172) (Abclonal, AP0116), anti-ULK1 (Proteintech, 20986-1-AP), anti-p-ULK1(Ser555) (CST, S555), anti-ATP1B2 (Proteintech, 22338-1-AP), anti-GAPDH (Proteintech, 60004-1), and anti-β-actin (Proteintech, 60008-1). DAPI (Sigma-Aldrich, D9542), FITC-labeled goat anti-rabbit IgG (H + L) (Beyotime, a0562), and Cy3-labeled goat anti-mouse IgG (H + L) (Beyotime, a0521) were also used.

2.2. Patients

Fibrotic liver biopsy specimens (fibrosis stage: F3-4) were obtained from 9 patients with liver fibrosis due to bile duct stones (9 cases). Normal liver specimens were obtained from 6 patients who underwent a partial liver resection for hepatic hemangioma. All patients signed the informed written consent, and the Ethics Committee at the local hospital approved the use of samples.

2.2.1. Animal experimental design

Sprague-Dawley (SD) rats and C57 mice were provided by the Laboratory Animal Center (Southern Medical University, China) and were approved by the Committee on the Ethics of Animal Experiments of Southern Medical University. Animals were housed under a 12:12 h light/dark cycle at 22–24 °C.

2.2.2. BDL-induced liver fibrosis rat model

Male SD rats (180–220 g) were subjected to BDL (n = 72) or a sham operation (n = 16) for 28 days. At Days 3, 6, 9, 12, 15, 18, 21, 24, and 28, BDL-induced rats were randomly sacrificed (n = 4 per group). Alternatively, BDL-induced rats were randomly divided into a BDL group (n = 12 for 18 days and 28 days) and two therapy groups: co-treatment with spironolactone (40 mg/kg•d, gavage, n = 12 for 18 days and 28 days), 3MA (10 mg/kg•d, intraperitoneal injection, n = 12 for 18 days and 28 days).

2.2.3. Hyperaldosteronism (Aldosterone-Salt) model

In total, 36 male C57 mice (18–22 g) were randomly divided into five groups (vehicle-control, Aldosterone-Salt, and administration with spironolactone or 3MA; n = 9 per group). All mice were fed with 1% NaCl for 28 days. The Aldosterone-Salt and the two treatment groups were treated with aldosterone (0.1 μg/g h) continuously via osmotic mini-pumps for 28 days, while the two therapy groups were co-treated with spironolactone (40 μg/g d, gavage), 3MA (15 μg/g d, intraperitoneal injection).

2.3. Measurement of serum aldosterone

The serum aldosterone was detected by an aldosterone ELISA Kit (Elabscience, E-EL-0070c), according to the manufacturer instructions. The results were read and calculated by ELIASA.

2.4. Histological analysis and immunohistochemistry

Paraffin sections (4 μm) of animal and human liver tissues were prepared with hematoxylin and eosin (H & E) staining and Sirius Red staining. Immunohistochemical detection of α-SMA and vWF, were performed on paraffin sections (4 μm), and subsequent sections were exposed to HRP-antibody colored with DAB, and visualized by microscopy (BX51, Olympus, Japan). The degree of liver fibrosis and the number of α-SMA- or vWF-positive cells were quantified with Image J software.

2.5. Fluorescence staining

Paraffin sections (4 μm) were prepared for immunofluorescence, incubated with primary antibody overnight, followed by the secondary antibody, and then mounted with DAPI. The primary antibodies included anti-CD31 (1:200), anti-vWF (1:50), anti-MR (1:200), and anti-

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