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Catalase inhibition induces pexophagy through ROS accumulation

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ABSTRACT

Peroxisomes are dynamic and multifunctional organelles involved in various cellular metabolic processes, and their numbers are tightly regulated by pexophagy, a selective degradation of peroxisomes through autophagy to maintain peroxisome homeostasis in cells. Catalase, a major peroxisome protein, plays a critical role in removing peroxisome-generated reactive oxygen species (ROS) produced by peroxisome enzymes, but the contribution of catalase to pexophagy has not been reported. Here, we investigated the role of catalase in peroxisome degradation during nutrient deprivation. Both short interfering RNA-mediated silencing of catalase and pharmacological inhibition by 3-aminotriazole (3AT) decreased the number of peroxisomes and resulted in the downregulation of peroxisomal proteins, such as PMP70 and PEX14 under serum starvation. In addition, treatment with 3AT induced NBR1-dependent autophagy and PEX5 ubiquitination in the absence of serum, which was accompanied by accumulation of ROS. Co-treatment with antioxidant agent N-acetyl-L-cysteine (NAC) prevented ROS accumulation and pexophagy by modulating peroxisome protein levels and the association of NBR1, a pexophagy receptor with peroxisomes. Taken together, these findings demonstrate that catalase plays an important role in pexophagy during nutrient deprivation.

1. Introduction

The peroxisome is a cellular organelle that plays an important role in various metabolic processes including β-oxidation of very long chain and branched chain fatty acids, synthesis of bile acids and ether lipids, and decomposition of hydrogen peroxide (H_2O_2) [1–3]. Peroxisome number is tightly controlled according to cellular needs. Under conditions of metabolic or cellular stresses, the number of peroxisomes is reduced through pexophagy [4–6]. Pexophagy is a specialized form of autophagy that degrades peroxisomes that is critical for maintaining cellular homeostasis [7,8]. Ubiquitination on the cytosolic face of peroxisomes is required for recruitment of the autophagy receptor proteins sequestosome (SQSTM)1/p62 and neighbor of BRCA1 gene (NBR)1, which bind to microtubule-associated proteins 1 A/1 B light chain (LC)3-associated autophagosomes for degradation [9–12]. Among peroxisome proteins, PMP70 and PEX5 are known to be ubiquitinated during pexophagy [13,14]. Furthermore, it has been reported that peroxisome E3 ligases PEX2, PEX10, and PEX12 are related to PEX5 ubiquitination for peroxisome degradation [14,15]. Since oxygen is consumed in various metabolic reactions in peroxisomes, oxygen availability able to regulate peroxisome number. Under hypoxic conditions, the peroxisome fails to function properly and pexophagy is induced to remove the damaged peroxisomes through hypoxia-inducible factor 2 alpha (Hif-2α) activation [16]. Also, it has been suggested that ROS triggers pexophagy through ATM activation [17].

Catalase, a porphyrin heme-containing enzyme is a classical marker protein of peroxisomes. Catalase decomposes H_2O_2 generated by various peroxisomal oxidases including acyl-CoA oxidase, urate oxidase, and xanthine oxidase [18,19]. Even though peroxisome contains several antioxidant enzymes, catalase plays a major role for protecting the adverse effects of accumulating peroxides

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[20,21]. Catalase inhibition deteriorated peroxisomal fatty acyl-CoA beta-oxidation by accumulated H_2O_2 in rodent liver [22]. Also, catalase protects the kidney under diabetic condition through maintaining peroxisome function [23]. In addition, the over-expression of catalase in mice extends lifespan of animals by reducing H_2O_2 production [24].

While the role of catalase on antioxidant effects has been intensively investigated, the relationship between catalase and pexophagy remains elusive. Here, we examined the role of catalase on pexophagy during serum starvation. Our data show that an inhibition of catalase activity increases peroxisome degradation through pexophagy during serum starvation. Also, catalase inhibition triggers the accumulation of ROS during starvation, which induces peroxisome loss through activation of selective autophagy.

2. Materials and methods

2.1. Cell culture, siRNAs and transient transfection

Retinal pigmented epithelial (RPE)1 cells (a gift from Dr. Joon Kim, KAIST, Korea) and HepG2 cells were cultured at 37 °C in a 5% CO_2 incubator and maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Small interfering (si)RNA against human catalase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA; sc-45330). HepG2 cells were transfected with the siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen). Cells were transiently transfected with pFLAG-PEX5 and pHA-ubiquitin plasmids using Lipofectamine 3000 (Invitrogen).

2.2. Western blot analysis

To determine the expression level of target proteins, western blotting was performed. Briefly, cells (3×10^6) were scraped off the culture plates and centrifuged at $1000 \times g$ for 5 min at 4 °C. They were then homogenized with RIPA buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM EDTA) mixed with 1X of protease and phosphatase inhibitor cocktail (GenDEPOT), centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was taken. After measuring protein concentration, cell lysate was boiled at 97 °C for 10 min and subject to immunoblot analysis using antibodies against catalase (Rockland, Gilbertsville, PA, USA), PEX5 (GeneTex, Irvine, CA, USA), PEX14 (Bethyl Laboratories, Montgomery, TX, USA), PMP70, LC3, FLAG, HA (Sigma-Aldrich, St. Louis, MO, USA), SQSTM1/p62 (Abnova, Taipei, Taiwan), and NBR1 (Proteintech, Chicago, IL, USA). After extensive washes, protein bands were visualized with an enhanced chemiluminescence detection system (AbFrontier).

2.3. Cell fractionation

Pooled HepG2 cell pellets were washed with ice-cold PBS, scraped and centrifuged at $1000 \times g$ for 5 min at 4 °C. Cell pellet was then homogenated in 400 μl of buffer A (50 mM HEPES pH 7.6, 1.5 mM $Mgcl_2$, 10 mM KCL) mixed with 1X of protease and phosphatase inhibitor cocktail. Homogenate was centrifuged at $1000 \times g$ for 10 min at 4 °C and the supernatant was transferred a new tube. Supernatant was centrifuged at $13{,}000 \times g$ for 30 min at 4 °C. After centrifugation, Supernatant (cytosol fraction) was transferred to a new tube and pellet (membrane fraction) was resuspended in RIPA buffer. Both fractions were used for immunoblot analysis [25].

2.4. Pexophagy assay using mRFP-GFP-SKL

RPE1 cells expressing the monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-serine-lysine-leucine (SKL) plasmid were seeded on coverslips in 12-well plates [26]. At 70% confluence, cells were treated with 10 mM 3-amino-1,2,4-triazole (3AT) or left untreated in the presence or absence of serum for 36 h. Cells were then washed with phosphate-buffered saline (PBS, pH 7.4), fixed in 4% paraformaldehyde at room temperature for 20 min, and washed with PBS. After mounting the coverslips, cells were observed under a fluorescence microscope. The number of cells with red puncta was counted and presented as a percentage of total cells.

2.5. Immunofluorescence

HepG2 cells were washed with PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich; HT5014) in PBS at room temperature for 20 min, and incubated in 0.1% Triton X-100 in PBS. After blocking in 3% bovine serum albumin for 1 h, cells were incubated with anti-PMP70 and -NBR1 antibodies (1:1000) at 4 °C. Cells were washed with PBS, labeled with Alexa Fluor-488 and Fluor-568 for 30 min, washed twice with PBS, and incubated with 10 mM 4′,6-diamidino-2-phenylindole (DAPI) in PBS at room temperature for 10 min. After mounting the coverslips, cells were observed under a confocal laser scanning system (Fluoview 1000; Olympus, Tokyo, Japan). Cells were scored in blinded fashion.

2.6. Measurement of ROS

ROS level was measured with the dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA). HepG2 and Chang/peroxi/Hyper-SKL (Chang) cells were cultured in the presence or absence of 3 A T with or without serum [27]. DCFDA (1 μM) was added to cells, followed by incubation for 45 min at 37 °C and 5% CO_2. Cells were then washed with PBS and observed under a fluorescence microscope.

2.7. Quantitative reverse transcription (qRT-)PCR

Total RNA was isolated from HepG2 cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was used for qRT-PCR analysis with SYBR Green (Roche Diagnostics, Berlin, Germany). The forward and reverse sequences of primers were as follows: 36B4, 5′-TGCATCAGTACCCCATTCTATCA-3′ and 5′-AAGGTGTAATCCGTCTCCACAGA-3'; and catalase, 5′-CTCAGGTGCGGGCATTCT-3′ and 5′-CAATGTTCTCACACA-GACGTTTCC-3'.

2.8. Immunoprecipitation

HepG2 cells were scraped off the culture plates and centrifuged at $1000 \times g$ for 5 min at 4 °C. They were then homogenized in 0.5 ml Seize 2 buffer (150 mM NaCl [pH 7.2], 50 mM Tris HCl) containing 0.2% Nonidet P-40 (Calbiochem, San Diego, CA, USA) mixed with $1 \times$ protease and phosphatase inhibitor cocktail. Detergent cell lysate was subjected to immunoprecipitation with anti-FLAG antibody along with 50 μl protein A/G agarose beads (Santa Cruz Biotechnology). After incubation, beads were collected by centrifugation, washed 3 times in 1 ml of Seize 2 buffer, resuspended in 100 μl of $1 \times$ SDS loading buffer, and boiled for 5 min. After centrifugation at $16{,}000 \times g$ for 3 min at room temperature, supernatant was transferred to a fresh tube, and aliquots of immunoprecipitants were subjected to immunoblot analysis with monoclonal anti-FLAG or anti-HA antibodies.