Aquatic Toxicology 194 (2018) 46–56

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

The role of the p38-activated protein kinase signaling pathway-mediated autophagy in cadmium-exposed monogonont rotifer *Brachious koreanus*

Hye-Min Kang[a,1], Chang-Bum Jeong[a,1], Min-Sub Kim[a], Jin-Sol Lee[a,b], Jiaying Zhou[a], Young Hwan Lee[a], Duck-Hyun Kim[a], Eunyoung Moon[c], Hee-Seok Kweon[c], Su-Jae Lee[d], Jae-Seong Lee[a,*]

[a] Department of Biological Science, College of Science, Sungkyunkwan University, Suwon 16419, South Korea
[b] Department of Chemistry, College of Science, Duksung Woman's University, Seoul 01369, South Korea
[c] Electron Microscopy Research Center, Korea Basic Science Institute, Daejeon 34133, South Korea
[d] Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 04763, South Korea

ARTICLE INFO

Keywords:
Autophagy
LC3 I/II
Mitogen-activated protein kinases
p38 MAPK
Rotifer

ABSTRACT

Autophagy is a 'self-eating' system that regulates the degradation of cellular components and is involved in various biological processes including survival and development. However, despite its crucial role in organisms, the regulatory mechanism of autophagy remains largely unclear, particularly in invertebrates. In this study, conserved autophagy in the rotifer *Brachionus koreanus* in response to cadmium (Cd) exposure was verified by measuring acidic vesicle organelles using acridine orange (AO) and neutral red (NR) staining, and by detecting LC3 I/II on Western blot and immunofluorescence. We also demonstrated activation of p38 mitogen-activated protein kinase (MAPK) in response to Cd-induced oxidative stress, leading to the induction of autophagy in *B. koreanus*. This was further verified by analysis of MAPK protein levels and immunofluorescence of LC3 I/II after treatment with reactive oxygen species (ROS) scavengers and inhibitors specific to MAPKs. We propose a p38 MAPK-mediated regulatory mechanism of autophagy in *B. koreanus* in response to Cd-induced oxidative stress. This study will contribute to a better understanding of autophagic processes in invertebrates and its modulation by environmental stressors.

1. Introduction

Autophagy is a well-known self-eating system that is responsible for the degradation of cytoplasmic components, thereby playing important roles in the maintenance of cellular functions (Mizushima, 2007; Ravikumar et al., 2010). This system mainly digests proteins that have an abnormal and/or unnecessary function for recycling materials and saving energy. Autophagic mechanisms are also induced to maintain homeostasis in response to stimuli such as nutrient starvation, hypoxia, and endoplasmic reticulum (ER) stress (Mizushima, 2005; Mizushima and Komatsu, 2011).

Since the first identification of autophagy-related (*ATGs*) genes in yeast (Klionsky et al., 2003), ortholog genes have been reported and characterized in many organisms from humans to lower organisms including nematodes (Meléndez and Neufeld, 2008; Meléndez and Levine, 2009). Among numerous *ATGs*, core *ATGs* including *ATG8* (LC3 I/II) are conserved across the animal taxa and are essential components of the autophagy machinery (Yang and Kilonsky, 2011; Shibutani et al., 2015). In invertebrates, only a few studies have been conducted on autophagy using model organisms including fruit flies and nematodes. In the nematode *Caenorhabditis elegans*, the role of core ATGs in autophagosome formation was demonstrated by knockdown of core *atg-7* and *atg-12* (Hars et al., 2007). In *Atg8a*-mutant *Drosophila*, a decrease in longevity was observed with accumulation of insoluble ubiquitinated proteins because a defect in Atg8a inhibits vesicle expansion for autophagosome formation (Simonsen et al., 2008). However, despite the vital functions of autophagy its role in invertebrates remains unclear, particularly for non-model species. Thus, in the present study we explored autophagic processes in the rotifer *Brachionus koreanus* to expand our knowledge of invertebrate autophagic processes.

Cadmium (Cd) is a widely distributed toxicant in the aquatic environment. Cd is easily transferred from low- to high-level organisms in the aquatic food web due to its high bioavailability, resulting in critical impacts on the aquatic ecosystem (Kay, 1985; Ruangsomboon and

* Corresponding author.
E-mail address: jslee2@skku.edu (J.-S. Lee).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.aquatox.2017.11.002
Received 16 October 2017; Received in revised form 6 November 2017; Accepted 8 November 2017
Available online 10 November 2017
0166-445X/

Wongrat, 2006). Moreover, regarding the high bioreactivity of Cd, generation of oxidative stress in an organism was reported in previous studies (He et al., 2008). In this context, autophagy could play a crucial defensive role under oxidative conditions by eliminating accumulated and damaged or abnormal materials, as oxidative stress triggers damage of biological molecules including DNA, proteins, and lipids (Lee et al., 2012; Liu et al., 1996; Sureshbabu et al., 2015). Among aquatic invertebrates, defense-related proteins such as heat-shock proteins and metallothioneins are activated as an early defensive response in the embryo of the sea urchin *Paracentrotus lividus* (Russo et al., 2003; Roccheri et al., 2004), whereas autophagy is initiated when the early defensive response is insufficient to maintain developmental processes in the presence of Cd-induced stress (Chiarelli et al., 2011; Agnello et al., 2007).

Rotifers are widely distributed spatially and numerically in the marine environment, playing important roles in the ecosystem as a primary consumer. They have several advantages for laboratory studies such as a short life cycle (~24 h), ease of incubation, and rapid reproduction (Dahms et al., 2011). Furthermore, the genome database for the rotifer *B. koreanus* has recently been established by Jae-Seong Lee's group (unpubulished data), facilitating in-depth molecular studies of this species.

In the present study, the induction of autophagy was verified in response to Cd exposure by detecting the marker protein of autophagy, LC3 I/II, with visualization of acidic vesicle organelles (AVOs). Moreover, the regulatory mechanism of mitogen-activated protein kinases (MAPKs) in Cd-exposed *B. koreanus* was investigated and revealed the involvement of p38 MAPK in autophagy induction in response to Cd-induced oxidative stress. This was further supported by immunofluorescent staining of LC3 I/II on whole-mount rotifers. To the best of our knowledge, this is the first study revealing the signaling pathway of autophagy in aquatic invertebrates.

2. Materials and methods

2.1. Chemicals

Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$; molecular weight 228.36, Sigma-Aldrich, St. Louis, MO, USA; purity > 98%), *N*-acetyl-L-cysteine (NAC; Sigma-Aldrich), SP203580 (Calbiochem, San Diego, CA, USA), SP60025 (Sigma-Aldrich), U0126 (Calbiochem), acridine orange (AO, Amresco, Solon, OH, USA), neutral red (NR, Sigma-Aldrich), rapamycin (InvivoGen, San Diego, CA, USA), bafilomycin A_1 (InvivoGen), and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, OR, USA) were used according to the instructions of the manufacturer.

2.2. Rotifer culture and Cd exposure

The monogonont rotifer *B. koreanus* was collected at Uljin (36°58′43.01″N, 129°24′28.40″E) in South Korea and reared in artificial sea water (ASW; TetraMarine Salt Pro, Tetra™, Cincinnati, OH, USA) with 15 practical salinity units (psu) under a 12:12 h light:dark photoperiod at 25 °C. The green microalga *Tetraselmis suecica* (Chlorophyceae) was cultured in a Walne's medium (Walne, 1970) at 20 °C with 24 h light exposure (4000 Lux). Rotifers were fed green microalga *Tetraselmis suecica* (Chlorophyceae) once a day (6×10^4 cells/ml). Species identification was performed by analysis of mitochondrial DNA cytochrome oxidase I (*CO1*) and morphologic characteristics (Hwang et al., 2013; Hwang et al., 2014; Mills et al., 2017).

For Cd exposure, adult rotifers (> 150 μm) were exposed to various concentrations of Cd (10 [43.79 μM], 20 [87.58 μM], 30 [131.37 μM], 40 [175.16 μM], and 50 mg/L [218.95 μM]) and to 30 mg/L Cd for different exposure times (1, 3, and 6 h) in 50 mL ASW. Cd was chosen as a model stressor in this study due to its critical impacts on animal health and ecosystem (reviewed by Järup and Åkesson, 2009). Rotifers were not fed during the exposure. All experiments were performed in triplicate.

2.3. Identification and alignment of LC3 genes

The amino acid sequence of the LC3 gene in *B. koreanus* was identified from the whole-genome database of *B. koreanus* (unpublished data; total scaffold nos. 1087; total length, 110 Mb; N50 value, 1.09 Mb) using similar gene sequences from human as a query. Local BLAST search (BLASTp) was conducted with the fully assembled transcripts against the non-redundant (NR) database of the NCBI. BLASTp hits with an E-value $\leq 10^{-5}$ were considered significant.

B. koreanus LC3 and human LC3 were aligned using MEGA software (ver. 6.0; Center for Evolutionary Medicine and Informatics, Tempe, AZ, USA) with the ClustalW alignment algorithm (Thompson et al., 1997) to analyze the epitope site of LC3 antibody (Sigma-Aldrich).

2.4. Rapamycin and bafilomycin A_1 treatment

To investigate the functional conservation of autophagy in *B. koreanus*, rapamycin and bafilomycin A_1 were used as an activator and inhibitor of autophagy, respectively. LC3 I/II was detected as a marker protein of autophagy (Tanida et al., 2008). Conserved autophagy in *B. koreanus* was investigated by analyzing the level of LC3 I/II in response to 1 μM rapamycin for 1, 3, and 6 h. In addition, 400 nM bafilomycin A_1 was used to verify the inhibition of autophagy.

2.5. Acridine orange (AO) and neutral red (NR) staining

AO staining was conducted to observe AVOs using whole-body mounts of rotifers. Rotifers (~300) were exposed to different concentrations of Cd (10, 20, 30, 40, and 50 mg/L) for 24 h, incubated with 2 μg/mL AO for 10 min, and subsequently washed three times with ASW. AO fluorescence was detected using confocal laser scanning microscopy (CLSM; LSM 510 META; Zeiss, Oberkochen, Germany) with a 20 × lens. The excitation and emission wavelengths were 473 and 520 nm for green fluorescence and 559 and 572 nm for red fluorescence, respectively. Red and green fluorescence ratio was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

NR staining was performed under the same Cd exposure conditions as AO staining. Rotifers exposed to Cd were incubated in 100 mM NR solution for 5 min and washed three times with ASW. Rotifers were observed using a fluorescence microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan) and the red stained areas were quantified using Image J software.

2.6. Immunofluorescence

Immunofluorescence assays were performed as described in Kotikova et al. with minor modifications (Kotikova et al., 2005). Briefly, rotifers were exposed to chemicals according to the experiment and fixed with 4% paraformaldehyde for 5 min. After washing three times in PBS-T, the fixed samples were incubated in blocking buffer (0.5% BSA in PBS-T) for 1 h. Rotifers were transferred to primary antibody solution (1:500 diluted in blocking buffer) for 48 h at 4 °C and washed three times in the washing buffer for 1 h. Washing buffer was replaced with FITC-labeled goat anti-rabbit IgG antibody (Sigma-Aldrich; 1:200 diluted blocking buffer) and incubated for 24 h at 4 °C. Rotifers were observed using FITC (488 nm/520 nm wavelengths) by confocal laser scanning microscopy with a 20 × lens.

2.7. Transmission electron microscopy

Rotifers were initially fixed for 1 h with 2.5% glutaraldehyde in 0.1 M phosphate (pH 7.3) at room temperature. After the fixation, the