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The protective role of multixenobiotic resistance (MXR)-mediated ATP-binding cassette (ABC) transporters in biocides-exposed rotifer *Brachionus koreanus*

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ABSTRACT

P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP) are ATP-binding cassette (ABC) transporters that confer multixenobiotic resistance (MXR) via their efflux activity, which enables a variety of xenobiotics to be expelled from cells. MXR has been proposed as the first line of defense against xenobiotics. In this study, the protective roles of P-gp and MRP in the rotifer *Brachionus koreanus* were examined in response to four biocides (alachlor, chlorpyrifos, endosulfan, and molinate) using fluorescent substrates and inhibitors specific to P-gp and MRP. Exposure of rotifers to the four biocides resulted in increased P-gp and MRP activity. Moreover, the rotifers became more sensitive to the biocides with a reduced tendency in survival and slower population growth rates, when P-gp or MRP was inhibited. These findings suggest that P-gp and MRP are involved in the defense system in response to biocide exposure. Furthermore, the transcriptional levels of the genes encoding P-gp and MRP were examined to uncover the mechanism by which MXR is regulated. Our results demonstrate a crucial role of the MXR efflux system in the defense response to biocides, thereby providing a better understanding of rotifer defense mechanisms on the molecular level.

1. Introduction

In aquatic organisms, cellular membranes act as the final physical barrier to xenobiotics, since the membranes are in constant contact with the ambient water column that contains various anthropogenic pollutants. In this respect, the efflux activities of membrane transporters can be considered as the first line of defense to xenobiotic exposure in aquatic organisms. Among the transporters, ATP-binding cassette (ABC) transporters hydrolyze ATP as an energy source and actively transport multiple substrates across the cellular membrane against a concentration gradient. Since the first identification of P-glycoprotein (P-gp) as an efflux transporter mediating multidrug resistance (MDR) in cancer cell lines (Juliano and Ling, 1976), subsequent identification of primary efflux transporters, namely multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) (Leslie et al., 2005; Robey et al., 2009), shed light on the mechanisms of multixenobiotic resistance (MXR) (Deeley et al., 2006). Since these ABC transporters have wide substrate spectra and are conserved across animal taxa, their potential role in aquatic invertebrate defense against environmental pollutants has been highlighted in several studies. For example, conserved efflux function of P-gp was demonstrated in the marine copepod *Tigriopus japonicus*. Moreover, P-gp activity was shown to be increased in response to metal exposure (cadmium, copper, and zinc), accompanied by transcriptional and translational up-regulation (Jeong et al., 2014). In the rotifer *Brachionus koreanus*, the efflux activity and transcriptional level of P-gp were shown to be significantly increased in response to several pharmaceutical stimuli (Rhee et al., 2012). In addition, it was recently revealed that the *ABC* genes, which mediate the MXR phenotype in aquatic invertebrates, are highly diversified via lineage-specific gene expansion (reviewed by Jeong et al., 2017b). This finding implies that MXR-mediated ABC transporters have an important role in aquatic invertebrates. However, the mechanisms by which these transporters are involved in defense against environmental pollutants in aquatic invertebrates are still unknown. Thus, to evaluate the defensive function of efflux transporters in aquatic invertebrates, we investigated the role of MXR-mediated ABC transporters in the response to biocides in the rotifer *B. koreanus*.

Biocides have been extensively used for several decades to control

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crop pests and to prevent vector-borne diseases. Since the ocean is the ultimate destination of anthropogenic pollutants, biocides are released continuously into the marine environment. These biocides have serious negative impacts on the aquatic ecosystem, since they are also toxic to their non-target aquatic organisms (Goldberg, 1995; Konstantinou et al., 2006). In particular, biocides have been shown to cause oxidative stress and to have endocrine-disrupting effects on aquatic organisms. For example, in mollusks, imposex and intersex are induced as a result of the endocrine-disrupting effects of tributyltin (Matthiessen and Gibbs, 1998). In the rotifer *B. koreanus*, the intracellular reactive oxygen species (ROS) level was shown to be increased in response to several biocides, indicating the generation of oxidative stress by exposure to biocides (Kim et al., 2015). To date, only a few studies have investigated the mechanism by which aquatic invertebrates defend themselves from biocides, although a number of studies have revealed that biocides have adverse effects on aquatic invertebrates.

Rotifers are considered suitable species for ecotoxicological studies for several reasons (Dahms et al., 2011; Snell and Marcial, 2017; Won et al., 2017). First, rotifers play an important role in the aquatic ecosystem since they connect the energy flow from producers to higher trophic level organisms via the prey-predator relationship. Considering the ecological niche of rotifers as an important food source to predators, they are the vehicles of bioaccumulation and/or biomagnification of environmental pollutants in secondary higher trophic level organisms. Moreover, rotifers are small (≈150–200 μm) and exhibit a short generation cycle (≈24 h), simple structure, genetic homogeneity, high fecundity, and easy laboratory maintenance (Snell and Janssen, 1995; Dahms et al., 2011).

In this study, the rotifer *B. koreanus* was used as a model species to investigate the role of MXR-mediated ABC transporters in the response to biocides. The activities of P-gp and MRPs were examined. In addition, the transcriptional levels of P-gp and MRPs were examined in response to four different biocides; alachlor, chlorpyrifos, endosulfan, and molinate. To determine whether the MXR-mediated ABC transporter is essential for survival of biocide-exposed rotifers, rotifers were exposed to biocides in the presence or absence of specific inhibitors for P-gp or MRP, and rotifer mortality was assessed. Our study sheds light on the first line defense mechanism in aquatic invertebrates in response to biocides.

2. Materials and methods

2.1. Rotifer culture

The monogonont rotifer *B. koreanus* was collected at Uljin (36°58′43.01″N, 129°24′28.40″ E) in South Korea. A single individual rotifer was isolated, reared, and maintained in filtered artificial seawater (TetraMarine Salt Pro, Tetra, Cincinnati, OH, USA). The strain was maintained by serial transfer of asexual populations at 25 °C under a light:dark 12:12 h photoperiod at 15 practical salinity units (psu). The green alga *Chlorella* sp. was used as a live diet (approximately 6×10^4 cells/mL). Species identification was confirmed by morphological analysis and sequencing of the mitochondrial DNA gene *CO1* (Hwang et al., 2013; Hwang et al., 2014; Mills et al., 2016).

2.2. P-gp and MRP activities

To examine the putative function of multixenobiotic transporters in *B. koreanus*, the activities of P-gp and MRP were assessed by measuring the accumulation of fluorescent P-gp and MRP substrates (rhodamine B and calcein AM for P-gp and MRP, respectively) in response to biocide exposure. All exposure concentrations of biocides used in the present study were chosen based on published no-observed-effect concentration (NOEC) values (Kim et al., 2015).

Live rotifers (≈1000) were exposed to each of alachlor (300 μg/L [1.112 μM]; purity > 99%; Sigma-Aldrich, St. Louis, MO, USA), chlorpyrifos (500 μg/L [1.426 μM]; purity > 99%; Sigma-Aldrich), endosulfan (100 μg/L [0.246 μM]; purity > 99%; Sigma-Aldrich), and molinate (3000 μg/L [16.017 μM]; purity > 99%; Sigma-Aldrich) for 3 h in the presence of 2 μM rhodamine B for P-gp and 1 μM calcein AM for MRP. Then, the rotifers were washed with clean artificial seawater (ASW) and incubated again with the biocides for 1 h to observe the maximal effect on the transporter activity. After this incubation, the rotifers were washed with phosphate-buffered saline (PBS) and homogenized with a Teflon homogenizer. The resultant homogenates were centrifuged at 10,000 rcf for 10 min, and the supernatant was used for fluorescence measurement and protein quantification. Approximately 0.2 mL of supernatant from each sample was transferred to a 96-well black plate, and the fluorescence of the supernatant was measured using a Varioskan Flash instrument (Thermo Electron, Vantaa, Finland). Calibration curves of rhodamine B (excitation 535 nm; emission 590 nm) and calcein AM (excitation 485 nm; emission 535 nm) were used for quantitation. The measured fluorescence values were normalized to the total level of protein in each sample, as determined using the Bradford method (Bradford, 1976). All experiments were performed in triplicate, and the temperature was maintained at 25 °C.

2.3. In vivo experiments

To examine the inhibitory effects of the MXR phenotype in rotifer defense against biocides, survival and population growth rates were measured in the presence or absence of an inhibitor. Verapamil and MK571 were used as an inhibitor for P-gp and MRP, respectively, as those chemicals are the well-established agents to inhibit P-gp and MRP activities (Tsuruo et al., 1983; Gekeler et al., 1995; Ford, 1996).

For measurement of survival rate, 10 rotifers were transferred into 4 mL ASW in each well of a 12-well culture plate (SPL, Seoul, South Korea). Each well contained 300 μg/L alachlor, 500 μg/L chlorpyrifos, 100 μg/L endosulfan, and 3000 μg/L molinate in the presence or absence of 10 μM verapamil (Sigma-Aldrich, St. Louis, MO, USA) or MK571 (Sigma-Aldrich) as a specific inhibitor for P-gp or MRP, respectively. Rotifers were incubated in the exposure medium for 24 h at 25 °C, after which the numbers of dead and live rotifers were counted under a stereomicroscope (SZX-ILLK200, Olympus Corporation, Tokyo, Japan).

Rotifer neonates (hatched within 2 h) were collected from a pool of rotifer eggs and used to determine the population growth rate. An individual neonate was transferred into each well of a 12-well culture plate (SPL) under the same exposure conditions as those used for survival rate measurement. Rotifer populations were counted daily under a stereomicroscope (SZX-ILLK200) for 8 days.

During all experiments, the green alga *Chlorella* sp. (approximately 6×10^4 cells/mL) was supplied as a diet; approximately 50 percent of the testing solution was renewed daily. Three biological replicates were performed for each experiment.

2.4. Inhibitory effects of P-gp and MRP

To further examine whether the observed adverse effects in the *in vivo* experiments were due to MXR inhibition, the activities of P-gp and MRP were measured in response to biocides in the presence of a P-gp-specific or MRP-specific inhibitor. Briefly, live rotifers (≈1000) were exposed to alachlor (300 μg/L), chlorpyrifos (500 μg/L), endosulfan (100 μg/L), and molinate (3000 μg/L) for 3 h with the appropriate fluorescent substrate (2 μM rhodamine B for P-gp and 1 μM calcein AM for MRP) in the presence of 10 μM verapamil or MK571 as a P-gp-specific and MRP-specific inhibitor, respectively. Then, the rotifers were washed with clean ASW and incubated again with the biocides, with or without the inhibitor, for 1 h to observe the maximal effect on the transporter activities. After incubation, the rotifers were washed with PBS and observed under a fluorescence microscope under 100x magnification (Olympus IX71; Olympus Corporation, Tokyo, Japan).