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Tubulin posttranslational modifications induced by cadmium in the sponge *Clathrina clathrus*

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ABSTRACT

As sessile filter feeders, sponges are exposed to environmental stress due to pollutants of both anthropogenic and natural origins and are able to accumulate harmful substances. Thus, sponges are considered a good tool for the biomonitoring of coastal areas. In this study, we used biochemical and immunocytochemical analyses to provide new data on the cadmium-related changes in sponge cells. In particular, we analyzed the effects of different concentrations of cadmium on the microtubule network in the calcisponge *Clathrina clathrus*. Quantitative densitometry of the immunoblots showed that, while the levels of α- and β-tubulin remained relatively constant in *C. clathrus* when exposed to 1 and 5 μM $CdCl_2$, there were progressive shifts in the levels of some tubulin isoforms. Exposure for 24 h to sublethal concentrations of cadmium reduced the level of tyrosinated α-tubulin and enhanced the levels of acetylated and detyrosinated α-tubulin relative to the levels in controls. Confocal microscopy analysis of immunolabeled tissue sections showed that the inhibitory effect of cadmium was associated with a decrease in the labeling of the cells with a monoclonal antibody that recognizes tyrosinated α-tubulin. By contrast, the reactivity with a monoclonal antibody that recognizes acetylated α-tubulin and with a polyclonal antibody specific for detyrosinated α-tubulin was enhanced at the same time points. Because the acetylation and detyrosination of α-tubulin occur on stable microtubules, the marked enhancement of α-tubulin acetylation and detyrosination in Cd^{2+}-treated cells indicates that divalent Cd ions stabilize microtubules. The possibility that Cd^{2+} may increase the stability of cytoplasmic microtubules was tested by exposing Cd^{2+}-treated cells to a cold temperature (0 °C). As shown, the microtubule bundles induced by Cd^{2+}, which were labeled by the monoclonal antibodies against acetylated and detyrosinated α-tubulin, were resistant to cold.

1. Introduction

Metal pollution in aquatic environments is of a major concern and its effects are widely studied because they determine a cascade of events in trophic networks constraining physiological performances of organisms. Sponges, like other sessile active filter feeders, play relevant functions in the autodepurative processes of a wide array of aquatic ecosystems (Reiswig, 1974; Gili and Coma, 1998). They are able to accumulate harmful organic and inorganic substances dissolved or suspended in the water column, as a result of their active water pumping by choanocytes and filtering activity by phagocytic processes (Zahn et al., 1981; Verdenal et al., 1990; Hansen et al., 1995; Philp, 1999; Pérez et al., 2002, 2003, 2005; Cebrian et al., 2003; Ramoino et al., 2011; Ledda et al., 2012). As sponges are exposed to environmental hazards, they are useful biomonitors of pollution levels of both anthropogenic and natural origins (Reiswig, 1974; Gili and Coma, 1998; Cebrian et al., 2007; Bell, 2008).

Cadmium (Cd^{2+}) is among the most toxic and dangerous pollutants. It is a mineral widely used in the steel industry, in plastics and as a component of batteries, and it accumulates in the environment due to its extremely long half-life (Cole and Volpe, 1983; Crompton, 1997; Herber, 2004). It enters cells, binds to a multitude of molecules, interferes with transport across cell membranes and may therefore disturb homeostasis and functional performance of cells. In mammals, Cd^{2+} affects cell proliferation, differentiation,

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apoptosis and other cellular activities, and thus can cause numerous molecular lesions that may be related to carcinogenesis (for reviews, see Goering et al., 1995; Pinot et al., 2000; Huff et al., 2007; Thévenod, 2009; Moulis, 2010). In sponges the exposure to Cd^{2+} may induce apoptosis and the expression of stress proteins HSP70 and GRP78 as well as morpho-functional cellular changes as highlighted in *Suberites domuncula* and *Scopalina lophyropoda*, respectively (Wagner et al., 1998; Schröder et al., 1999; Cebrian and Uriz, 2007). Up to now, the effect of cadmium on cytoskeletal functions and, in particular, on microtubule assemblage has not been considered in marine invertebrates including sponges.

Microtubules play key functional roles in cell division, the intracellular trafficking of macromolecules and organelles, the beating of cilia and flagella and the dynamic organization of cell's morphology and architecture. Microtubule formation and disappearance are described by the dynamic instability theory, whereby individual microtubules exist in alternating stages of rapid disassembly and assembly processes (Mitchison and Kirschner, 1984; Sammak et al., 1987; Tran et al., 1997). Although most microtubules in a cell are dynamic structures with a relatively short half-life, subpopulations of less dynamic, more stable microtubules have also been identified in many cell types (Sammak et al., 1987; Schulze and Kirschner, 1987; Webster and Borisy, 1989). Stable microtubules resist depolymerization by antimitotic drugs and cold, frequently undergoing extensive tubulin posttranslational modifications during their long life (MacRae, 1997). These modifications include acetylation of α-tubulin, phosphorylation of β-tubulin, detyrosination of α- and rarely β-tubulin, the related Δ2-α-tubulin modification, readdition of tyrosine to α-tubulin, polyglutamylation and glycosylation of α- and β-tubulins, and palmitoylation (reviewed in Barra et al., 1988; Greer and Rosenbaum, 1989; Eddé et al., 1990; Bulinski and Gundersen, 1991; MacRae, 1997; Luduena, 1998; Rosenbaum, 2000; McKean et al., 2001; Hammond et al., 2008; Wloga and Gaertig, 2010). Several studies have demonstrated that the acetylation and detyrosination of α-tubulin occur on stable microtubules (Piperno et al., 1987; Webster et al., 1987; Bulinski et al., 1988; Webster and Borisy, 1989). Most of these modifications have been observed in a wide range of taxa from Protista to Metazoa.

Distinct patterns of cytoskeletal injuries in animals and plants could be induced by cadmium ions. The disassembly of the cytoplasmic microtubule network (Perrino and Chou, 1986) as well as the depolymerization of microtubules and the aggregation of actin filaments have been observed in cultured Swiss mouse 3T3 cells (Li et al., 1993). Cd^{2+} also affects the mechanisms controlling the organization of the microtubule cytoskeleton and the tubulin assembly/disassembly processes, inducing the formation of microtubule arrays, in interphase and mitotic cells from *Allium sativum* (Xu et al., 2009). Depending on the Cd^{2+} concentration and on the duration of exposure, the microtubules disintegrate into short rod-shaped fragments or they completely disappear in interphase cells of the green alga *Spirogyra decimina* (Pribyl et al., 2005).

In this study we show that, in the calcisponge *Clathrina clathrus*, Cd^{2+} stimulates a concentration-dependent increase in the levels of acetylated and detyrosinated α-tubulins. Furthermore, we show that microtubules of Cd^{2+}-treated cells are resistant to depolymerizing agents by exposing sponges to cold.

2. Material and methods

2.1. Materials

Specimens of the marine sponge *Clathrina clathrus* were collected by SCUBA diving at the Portofino Promontory (Ligurian Sea, Northwestern Mediterranean) at a depth of 15–25 m. Sponges where immediately transported to the laboratory in buckets containing seawater kept at 16 °C during transfer. The sponges were maintained in an aquarium at 16 °C containing filtered natural seawater until the start of the cadmium exposure experiment.

2.2. Incubation

To expose *C. clathrus* to cadmium, the collected sponge specimens were cut into fragments of *ca.* 2 cm^3 and were put into flasks containing 200 ml of filtered seawater supplemented with or without different concentrations of cadmium chloride (1 or 5 μM, corresponding to 0.18 or 0.91 mg/L, respectively) at 16 °C. Seawater was continuously oxygenated by pulses from an aquarium aerator. The sponge fragments were incubated for 24 h.

After 24 h of cadmium exposure, portions of the sponge fragments were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 6–12 h, rinsed in PBS, dehydrated and embedded in paraplast. Serial sections 5 μm thick were obtained and mounted on polylysine-coated slides.

Portions of the sponge fragments were utilized for cell isolation. After a clean cross cut was made to the sponge body with a sharp blade, the solution containing the cells was gently collected from the cut surface using a pipette. This material was then processed for immunoblotting and cell viability determination.

To examine the microtubule stability, some of control and Cd^{2+}-treated sponge cubes were washed with seawater and then further incubated on ice (0 °C). After 90 min of exposure, the sponge cubes were treated as described above (fixed, dehydrated, embedded in paraplast and sectioned) for immunofluorescence staining or processed for immunoblotting assay to monitor alterations in the organization of acetylated, detyrosinated and tyrosinated α-tubulin in microtubules.

For each set of experiments (exposure to cadmium and control), fragments from the same individual sponge were used. The experiments were replicated five times over several weeks.

2.3. Cd^{2+} and cell viability

The sponge cell viability after exposure to Cd^{2+} was determined using propidium iodide (PI) staining. Cells with intact plasma membranes are impermeable to PI, but PI complexes with the DNA of cells with damaged membranes, which results in cells with highly fluorescent nuclei (Vornov et al., 1991). A stock solution was prepared by dissolving 1 mg of PI in 1 ml of demineralized water, and 10 μl of PI stock solution was added to 1 ml of each cell suspension (final concentration: 10 μg/ml). The cells were counted under a microscope after 5 min.

2.4. Antibodies

Monoclonal anti-α-tubulin (clone B512, 1:2000), anti-β-tubulin (clone TUB 2.1, 1:200), anti-acetylated tubulin (clone 6-11B-1, 1:300), and anti-tyrosinated tubulin (clone TUB-1A2, 1:500) antibodies were obtained from Sigma Chemical Co (St. Louis, MO, USA). The polyclonal antibody specific for detyrosinated tubulin (1:200) was from Chemicon, Millipore (Darmstadt, Germany), and the secondary anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 488 antibodies were from Molecular Probes, Invitrogen (Carlsbad, CA, USA). The antibodies used were generated against mammalian tubulins. However, due to the structural conservation of tubulins (Gamulin et al., 2000), these antibodies also recognize, as we have checked, sponge tubulins.