Toxicology Letters 224 (2014) 356–361

Contents lists available at ScienceDirect

Toxicology Letters

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

Kinesin-dependent motility generation as target mechanism of cadmium intoxication

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HIGHLIGHTS

- Cadmium ions attack the kinesin/microtubule dependent transport system.
- Cadmium ions exert an inhibitory effect on microtubule formation.
- Cadmium ions cause a strong inhibition of the ATPase activity of kinesin.
- Cadmium ions slow down the movement of kinesin along microtubules.

ARTICLE INFO

Article history:
Received 23 September 2013
Received in revised form 1 November 2013
Accepted 4 November 2013
Available online 12 November 2013

Keywords:
Cadmium
Kinesin
ATPase
Motility
KIF5A
Tubulin

ABSTRACT

The anterograde vesicle transport within neurons critically depends on microtubules and on the activity of kinesin. The present study demonstrates that cadmium ions inhibit the *in vitro* assembly of microtubules from tubulin, whereby at high cadmium levels (~500 μM) unstructured protein aggregates were formed. Cadmium ions also significantly lower both the ATPase and motility activity of neuron-specific kinesin KIF5A in concentration-dependent manner. For the inhibition of KIF5A ATPase activity, an IC_{50} value of 10.4 ± 1.5 μM was determined. Inhibition could be widely compensated by addition of EGTA, but not by addition of thiols. The inhibitory effect of cadmium on KIF5A was considerably weakened by increasing ATP concentration. As nucleoside triphosphate binding is known to be accompanied by conformational changes within the kinesin motor domain, it might be suggested that these changes protect the motor domain against cadmium. The effects of cadmium ions on the kinesin–microtubule motility generating system are considered to contribute to the development of neuronal disorders caused by cadmium intoxication.

1. Introduction

Cadmium is a heavy metal, which accumulates in industrial and agricultural products. Actually, an evident tendency of elevation of environmental levels of cadmium in soil, water, and living organisms can be observed (Alexander et al., 2009). Beside cigarettes, food is a main source of cadmium uptake for human population. Cadmium uptake has been proved to be a serious health hazard, causing heavy damages of kidney, lung, liver, bone, and other organs (Godt et al., 2006).

Recently, there is an increasing number of reports on toxic effects of cadmium on the neuronal system. So, cadmium exposure was discussed to cause neuropsychological disorders (Hart et al., 1989; Sarchielli et al., 2012) as e.g., amyotrophic lateral sclerosis (Bar-Sela et al., 2001) or the myalgic encephalomyelitis/chronic fatigue syndrome (Pacini et al., 2012). Moreover, cadmium has become known to damage striatum (O'Callaghan and Miller, 1986). Also Parkinson disease might be related to cadmium exposure (Okuda et al., 1997).

The molecular mechanisms involved in neurotoxicity of cadmium are poorly understood. Oxidative stress seems to be one trigger for cadmium toxicity in tissues, including brain (Kumar et al., 1996). Cadmium can also affect proteasomal function and prion protein aggregation, which promote neurotoxicity (Kanthasamy et al., 2012).

Basic physiological functions of eukaryotic cells essentially depend on microtubules and on the activity of kinesin and dynein, which are ATPase-active microtubule-binding motor proteins playing key roles in the anterograde (Hirokawa and Noda, 2008; Vale et al., 1985) and retrograde (Schnapp and Reese, 1989) vesicle transport. Particularly, the communication between the cell body of neurons and their extended processes, based on the so-called axonal transport, uniquely requires the activity of motor proteins. There is growing evidence that any disturbances of the

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0378-4274/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2013.11.004

axonal transport, including mutations in kinesin genes, blocking kinesin–microtubule interaction, microtubule disruption, or inhibition of ATPase, might contribute to the development of neurodegenerative diseases (Chevalier-Larsen and Holzbaur, 2006; De Vos et al., 2008; LaPointe et al., 2013; Reid et al., 2002).

The effect of cadmium on the microtubule system has been discussed rather controversially. In optic nerves exposed to 200 μM cadmium ions, the microtubules were found to be disassembled (Fern et al., 1996). Moreover, cadmium salts were reported to inhibit microtubule formation *in vitro* (Liliom et al., 2000; Wallin et al., 1977). On the contrary, Brunner and co-workers did not find an inhibition of microtubule formation *in vitro* up to 1000 μM (Brunner et al., 1991).

So far, no data have been available concerning the influence of cadmium on kinesin. Therefore, besides re-evaluating the effect of cadmium ions on microtubule formation *in vitro*, the present study is focussed on the ATPase activity of neuron-specific kinesin and on kinesin-mediated motility generation in cell-free environment. The results obtained might contribute to better understanding molecular mechanisms of cadmium intoxication in common and of cadmium neurotoxicity in particular.

2. Materials and methods

2.1. Isolation of microtubule protein and microtubule formation

Microtubule protein was purified from porcine brain by two cycles of temperature-dependent disassembly/reassembly, according to principles described before (Shelanski et al., 1973; Vater et al., 1986), using a buffer containing 20 mM 1,4-piperazine diethane sulfonic acid (PIPES) (pH 6.8), 80 mM NaCl, 0.5 mM $MgCl_2$, 1 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA), and 1 mM dithiothreitol (DTT). The prepared microtubule protein contained about 85% tubulin and about 15% microtubule-associated proteins (MAPs), co-purifying with tubulin in stoichiometric ratio.

For microtubule formation protein stocks, stored at −80 °C, were diluted from 24.0 to 1.2 mg/ml (corresponding to about 10 μM tubulin) with the buffer lacking EGTA, transferred to glass cuvettes, and supplemented with GTP (0.5 mM final concentration) and cadmium chloride (final concentrations as indicated in Section 3). The resulting final concentration of EGTA was 50 μM. After shifting temperature to 37 °C, the kinetics of microtubule formation was recorded by measurement of turbidity at 360 nm (Gaskin et al., 1974) in a Cary 100 spectrophotometer (Agilent Technologies Deutschland GmbH), equipped with a temperature-controlled multichannel cuvette holder.

Corresponding control measurements without protein showed that cadmium chloride up to 500 μM did not change the turbidity signal compared to the cadmium-free sample.

2.2. Expression and purification of motor protein constructs

Human neuron-specific kinesin KIF5A (Niclas et al., 1994) was expressed in *Escherchia coli* as truncated construct containing amino acids 1–560 and purified as described formerly (Kalchishkova and Bohm, 2008), yielding a protein lacking artificial tags. The kinesin was adjusted in motility buffer (50 mM imidazole, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM dithiothreitol, pH 6.8) and stored at −80 °C.

2.3. ATPase activity measurement

The KIF5A was diluted 100-fold with ATPase assay buffer containing 50 mM PIPES and 5 mM $MgCl_2$ (pH 6.8). To measure the ATPase activity, the diluted KIF5A was mixed with paclitaxel-stabilized microtubules, reassembled from pure tubulin obtained by phosphocellulose column chromatography (Weingarten et al., 1975), and cadmium chloride (ultra dry, 99.999% Alfa Johnson Matthey GmbH, Germany). ATP hydrolysis was initiated by adding ATP (sodium salt, Roche Diagnostics GmbH, Germany). If not indicated otherwise, the resulting reaction mixture contained 1.5 μM tubulin, 168 nM KIF5A, 1.25 mM ATP, 0.5 μM dithiothreitol, and 30.4 μM EGTA. After 30-min incubation at 30 °C the reaction was stopped by addition of HCl (0.1 N final concentration). The ATPase activity was determined by measuring the released free inorganic phosphate, using a Malachite green staining technique (Martin et al., 1985). For this, Biomol Green™ Reagent (Enzo Life Sciences GmbH, Germany) was added according to instructions of the supplier, the samples (triple aliquots) were incubated 25 min in the dark, and the intensity of colour was read in a Cary 100 spectrophotometer at 650 nm. Calibration was done with a series of 20–80 μM disodium hydrogen phosphate using a commercial 800-μM standard solution (Enzo Life Sciences). It has been proved that cadmium chloride up to 1 mM (final concentration) does not affect phosphate determination under the assay conditions used (result not shown).

2.4. Microtubule gliding assay

Stock solutions of paclitaxel-stabilized microtubules, kinesin, ATP, and cadmium chloride and were transferred into ATPase assay buffer (kept at room temperature), resulting in final concentrations of 40 μg/ml tubulin, 0.5 mM ATP, 70 μM EGTA (arising from microtubule and kinesin preparations) and final concentrations of cadmium chloride and KIF5A as indicated in Results. To study the effect of EGTA, additionally 500 μM of the chelating agent were added. After 10-min preincubation at room temperature, 10-μl drops of this mixture were transferred onto microscopic 26 mm × 76 mm glass slides (Gerhard Menzel GmbH, Germany) pretreated with 5 mg/ml casein, covered with a 18 mm × 18 mm coverslip (Gerhard Menzel GmbH), and sealed with a mixture of vaseline, lanolin, and paraffin (Bohm et al., 2000a). The gliding microtubules were visualized by video-enhanced differential interference contrast microscopy (AVEC DIC microscopy) using an Axiophot microscope (Zeiss, Germany) and the image processes system Argus 20 (Hamamatsu Deutschland GmbH). Using the Argus 20 software, the gliding velocities were measured within the first 5 min after application of the kinesin–microtubule mixture onto the glass slide by tracing the leading end of microtubules over a distance of at least 10 μm. The arithmetic mean and SD were calculated from the data of at least 15 individual microtubules.

3. Results and discussion

The anterograde axonal transport in neurons essentially depends on the functional integrity of the interplay between the motor protein kinesin and microtubules. Disorders of the sensitive system of kinesin-mediated motility generation can result in disruption of the axonal transport, which seems to be a cause of the development of various neuronal diseases (Franker and Hoogenraad, 2013; Niwa et al., 2013), among them the hereditary spastic paraplegias (Ebbing et al., 2008; Ikenaka et al., 2012).

Tubulin and kinesin are known to be affected by different heavy metal ions, including mercury and lead (Bonacker et al., 2004, 2005; Thier et al., 2003). The central aim of the present study was to check whether cadmium ions affect main functional structural components of the motility-generating system underlying the anterograde vesicle transport.

Following this intention, at first microtubule assembly was recorded using the standard time-dependent turbidity measurement (Gaskin et al., 1974), in which the turbidity signal at 360 nm is a measure of the quantity of microtubules formed. We found that up to 50 μM cadmium chloride there were no remarkable effects. At 50 μM, the final turbidity level measured after 45 min was lowered by 15%; at 100 μM by 58.5% respectively (Fig. 1A). Surprisingly, at higher cadmium ion concentrations the turbidity levels determined at time point 45 min increased again.

Microtubules from mammalian brain are commonly known to be highly cold sensitive. Lowering temperature to 2 °C results in more or less complete disassembly of microtubules as seen for the control without cadmium chloride (Fig. 1A). However, at high cadmium chloride there was no turbidity decrease upon cooling (Fig. 1A). The reason for this behaviour might be the formation of cold-stable microtubules. However, the corresponding microscopic controls revealed that at 500 μM cadmium chloride no microtubules were formed (Fig. 2). Therefore, we conclude that the turbidity curves recorded in the presence of cadmium chloride are the result of superimposition of two counteracting processes: a cadmium-induced inhibition of microtubule assembly, expressed by lowered turbidity, and formation of unstructured protein aggregates (see Fig. 2C) at high cadmium, expressed by increased turbidity. Taking into account only the data measured for cadmium ion concentrations below 100 μM and excluding possible protein aggregate formation in this concentration range, an IC_{50} value of about 90 μM was crudely estimated.

Summarizing assembly measurements, our results confirm the report of Wallin et al. (1977). Using a viscosimetric assay to quantify microtubule formation, these authors determined an about 80% inhibition at 1000 μM cadmium chloride and 100 μM EGTA. In contrast, Brunner et al. (1991) reported that cadmium ions up