Archives of Biochemistry and Biophysics 560 (2014) 44–51

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Disrupted calcium homeostasis is involved in elevated zinc ion-induced photoreceptor cell death

Dadong Guo [a], Yuxiang Du [b], Qiuxin Wu [c], Wenjun Jiang [a], Hongsheng Bi [a,*]

[a] Shandong Provincial Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Therapy of Ocular Diseases, Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Therapy of Ocular Diseases in Universities of Shandong, Eye Institute of Shandong University of Traditional Chinese Medicine, Jinan 250002, China
[b] Shandong University of Traditional Chinese Medicine, Jinan 250002, China
[c] Affiliated Eye Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250002, China

ARTICLE INFO

Article history:
Received 24 April 2014
and in revised form 1 July 2014
Available online 19 July 2014

Keywords:
Zinc chloride
Calcium homeostasis
Plasma membrane calcium ATPase
Reactive oxygen species
Cell cycle arrest

ABSTRACT

Zinc (Zn), the second abundant trace element in living organisms, plays an important role in regulating cell metabolism, signaling, proliferation, gene expression and apoptosis. Meanwhile, the overload of Zn will disrupt the intracellular calcium homeostasis *via* impairing mitochondrial function. However, the specific molecular mechanism underlying zinc-induced calcium regulation remains poorly understood. In the present study, using zinc chloride ($ZnCl_2$) as a stressor, we investigated the effect of exogenous Zn^{2+} in regulating murine photoreceptor cell viability, reactive oxygen species (ROS), cell cycle distribution and calcium homeostasis as well as plasma membrane calcium ATPase (PMCA) isoforms (PMCA1 and PMCA2, i.e., ATP2B1, ATP2B2) expression. We found that the exogenous Zn^{2+} in the exposure range (31.25–125.0 μmol/L) results in the overgeneration of ROS, cell cycle arrest at G_2/M phases, elevation of cytosolic [Ca^{2+}], inactivation of Ca^{2+}-ATPase and reduction of both PMCA1 and PMCA2 in 661 W cells, and thus induces cell death. In conclusion, $ZnCl_2$ exposure can elevate the cytosolic [Ca^{2+}], disrupt the intracellular calcium homeostasis, further initiate Ca^{2+}-dependent signaling pathway in 661 W cells, and finally cause cell death. Our results will facilitate the understanding of cell death induced by the zinc ion-mediated calcium homeostasis disruption.

Introduction

Zinc (Zn),[1] which ranks second only to ferrum (Fe) as the most widely distributed transition metal in organisms, plays important roles in regulating metabolism, immune response, cell proliferation, differentiation, signaling pathway, gene expression, genomic stability and mitosis [1–8]. Meanwhile, Zn is an important cofactor in more than 300 cellular enzymes, which can exert its multiple biological functions through binding to extensively enzymes as well as to several classes of regulatory proteins to influence various organ functions [9,10], including zinc finger proteins and copper–zinc superoxide dismutase. All these characteristics of zinc peptidases and proteases can be attributed to zinc's inorganic chemical property as an effective Lewis acid.

Zn deficiency occurs in all age groups and nationalities [11], which causes a wide range of disorders, including immune insufficiency, infection, cholera, diarrhea, skin eruptions and dermatitis [12–14]. Consequently, Zn supplements, in most cases, can boost health and well-being. However, improper Zn supplements may also generate extremely dangerous side effects. For example, high concentrations of zinc can exert cytotoxic effect on target cells. It was reported that high concentration (0.5–10 mM) of Zn could cause hemoglobin aggregation and alteration in nanostructure of red blood cell membrane [15], and excessive extracellular free zinc (Zn^{2+}) can induce kidney proximal tubular cell damage through binding to the cell organelles and disrupting cellular processes [16]. In the meantime, Zn was reported to increase oxidative stress-induced apoptosis in an age-related fashion inhuman peripheral blood mononuclear cells [17]. Moreover, elevated intracellular Zn can result in AMPA receptor activation and thus lead to excitotoxic oligodendroglial death [18]. However, the detailed molecular mechanisms underlying zinc ion-induced calcium regulation have not been well understood.

Mitochondria are well known for being the eukaryotic cell powerhouses, and these organelles can participate in a wide range

* Corresponding author. Fax: +86 531 82861167.
E-mail address: azuresky1999@163.com (H. Bi).

[1] *Abbreviations used:* Zn, zinc; $ZnCl_2$, zinc chloride; ROS, reactive oxygen species; PMCA, plasma membrane calcium ATPase; Ca, calcium; Fe, ferrum; DMEM, Dulbecco's Modified Eagle Medium; MTT, colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; PBS, phosphate buffer saline; PI, propidium iodide.

http://dx.doi.org/10.1016/j.abb.2014.07.014
0003-9861/

of cellular functions involved in ATP synthesis, calcium regulation, generation of reactive oxygen species (ROS), and the intrinsic apoptotic pathway as well as steroid hormone biosynthesis [19]. ROS have been recognized as toxic aerobic metabolism byproducts that are harmful to cells. Generally, physiological levels of intracellular ROS are required to maintain genomic stability within cells, and environmental stresses can cause an enhanced production of ROS. The elevated ROS will damage DNA, proteins, lipids and other cell components even cause cell death. It has been shown that excessive ROS are closely associated with mitochondrial dysfunction [20–22], further disrupt the calcium homeostasis *via* activating Ca^{2+}-dependent signaling pathway, and finally induce cell death [23].

Calcium (Ca) is an important second messenger in eukaryotic cells that regulates many different cellular processes [24]. Under normal physiological circumstances, it is crucial for healthy cells to maintain low cytosolic free Ca^{2+} levels to perform the normal physiological functions. On the other hand, the disruption of intracellular calcium homeostasis will cause cell damage even death [25–28]. Thus, the intracellular calcium balance and homeostasis need a fine regulatory mechanism. Meanwhile, it is well known that membrane transporters and channel proteins are responsible for a proper functioning of the Ca^{2+}-sensitive signaling transduction pathways that can regulate cell growth and apoptosis [29,30]. Membrane transporters, including plasma membrane calcium ATPases (PMCAs), can expel Ca^{2+} from eukaryotic cells by means of the energy of ATP hydrolysis to maintain the low level of intracellular Ca^{2+} concentrations [31]. However, because of sulfhydryl, PMCAs, which would be inactivated under a prolonged oxidative stress circumstance, are susceptible to oxidative stress [32,33]. Accordingly, the regulatory role of PMCAs can be altered in maintaining calcium homeostasis.

PMCAs are high affinity Ca^{2+} pumps which play important roles in maintaining intracellular calcium homeostasis and signaling. Currently, there is now ample evidence that PMCAs have four isoforms, PMCA1-4 [34], and in which, PMCA2 (i.e., ATP2B2) is a calcium extrusion pump which exhibits a high affinity for calcium to make it particularly good at eliminating low to moderate levels of calcium [35,36]. It has been shown that PMCA2 is widely expressed in the mammalian central nervous system [37–39] while PMCA1 could be widely expressed in mouse photoreceptor cells, horizontal cells and cone bipolar cells which respond to light with a graded polarization [40].

A photoreceptor cell is a specialized type of neuron found in the retina that is capable of phototransduction. In the mouse retina, photoreceptor cells can receive the feedback signals from horizontal cells [41]. A 661 W cell line is a mouse SV-40 T antigen transformed photoreceptor cone cell line. As a kind of parenchymal cell line, 661 W cells are more susceptible to the alterations in outer microenvironmental conditions than other cell lines. As the main ingredient in eye drop, zinc has been applied in clinical treatment of conjunctivitis. To investigate the effect of extracellular free zinc ions on 661 W cells, we explored the alterations in cell viability, cell cycle distribution, ROS and cytosolic [Ca^{2+}], and PMCA1 and PMCA2 expression both mRNA and protein expression as well as their activities when cells exposed to different concentrations of $ZnCl_2$. The present study will facilitate the understanding of the cell death induced by disruption of intracellular calcium homeostasis mediated by the overload of Zn^{2+}.

Materials and methods

Cell culture

A murine photoreceptor cell line (661 W) was kindly provided by Eye Center of the Second Bethune Hospital, Jilin University, China. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life technologies, USA) in flasks (NEST Biotechnology, China) supplemented with 10% fetal bovine serum (HyClone, USA), 1.0 g/L of glucose, 100 U/mL of penicillin and 100 μg/mL of streptomycin, and were cultured at 37 °C in a water-saturated air incubator with 5% CO_2. Cell count was performed using an automated cell counter (TC10, Bio-Rad, USA).

MTT assay

To perform the MTT assay, 661 W cells in log phase were seeded in 96-well microtiter plates (NEST Biotechnology, China) and the initial density was 8.0×10^3 cells per well. After culture overnight, the supernatant of 661 W cells was discarded and 200 μL DMEM containing various concentrations (i.e., 31.25, 62.5, 93.75, 125.0, 187.5, 250.0, 375.0, 500.0 μmol/L) of $ZnCl_2$ (Sigma–Aldrich, USA) was added, and then cells were further cultured in a water-saturated air supplemented with 5% CO_2 at 37 °C for additional 24, 48 and 72 h, respectively. In the meantime, control cells ($ZnCl_2$-free cells) were also cultivated under the same conditions. Cell viability was evaluated by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is directly proportional to living cells. The result was expressed as follows: cell viability (%) = $A_{test}/A_{control} \times 100\%$, where A represents the relevant absorbance value at 490 nm, and 630 nm is used as reference wavelength. The results were obtained from three independent experiments performed in triplicate and were represented as mean ± SD (standard deviation), and the 50% inhibitory concentration (IC_{50}) was defined as the concentration required for 50% inhibition of cell growth.

Determination of intracellular ROS

To determine the effect of different concentrations of $ZnCl_2$ solution on the intracellular ROS level, the alterations in intracellular ROS level were measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Invitrogen, USA) by a flow cytometer (Accuri C6, USA).

Briefly, 661 W cells (4×10^5 cells per well) were seeded in a 6-well plate (NEST Biotechnology, China) and grown overnight, then the supernatant was discarded and cells were supplemented with 0, 31.25, 62.5 and 125.0 μmol/L of $ZnCl_2$ solution (final volume: 2 mL). Subsequently, cells were incubated at 37 °C for 2 h. Further, all cells were rinsed with phosphate buffer saline (PBS, Ca^{2+}- and Zn^{2+}-free, pH 7.4) twice and then digested with 0.25% trypsin. After harvest of 661 W cells, all cells were incubated with DCFH-DA solution (10 μM) in the dark at 37 °C for 30 min, washed with PBS, and then determined using flow cytometry within 30 min. The specific fluorescence signals that correspond to DCFH-DA were collected by means of a 525-nm band pass filter. As a rule, 1.0×10^4 cells were counted for each determination. This investigation was repeated three times and the result was expressed as mean ± SD.

Cell cycle distribution analysis

To investigate the cell cycle distribution, first, 661 W cells were treated with different concentrations of $ZnCl_2$ (0, 31.25, 62.5 and 125.0 μmol/L, respectively) in a 6-well plate (final volume: 2 mL per well) and cultured in a water-saturated incubator with 5% CO_2 at 37 °C for additional 12 h, and then 661 W cells were collected after digestion with 0.25% trypsin and washed with ice-cold PBS twice. Further, 661 W cells were resuspended in ice-cold 70% ethanol and kept at 4 °C overnight. After washing with ice-cold PBS twice and co-incubation with 50 mg/L propidium iodide (PI) (Beyotime, China), RNase (60 μg/mL) in the dark at 37 °C for 40 min, cell cycle distribution were finally determined by a flow