Toxicology 253 (2008) 110–116

Contents lists available at ScienceDirect

Toxicology

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

Suppressive effects of cadmium on neurons and affected proteins in cultured developing cortical cells

Ritsuko Ohtani-Kaneko[a,b,c,*], Hidekatsu Tazawa[d], Makoto Yokosuka[e], Minoru Yoshida[f], Masahiko Satoh[g], Chiho Watanabe[h]

[a] Department of Life Sciences, Toyo University, 1-1-1 Itakura, Oura, Gunma 374-0193, Japan
[b] Bio-Nano Electronic Research Centre, Toyo University, 2100, Kujirai, Kawagoe, Saitama 350-8585, Japan
[c] Graduate School of Interdisciplinary New Science, Toyo University, 2100, Kujirai, Kawagoe, Saitama 350-8585, Japan
[d] Department of Anatomy, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan
[e] Laboratory for Animal Neuroscience, Nippon Veterinary and Life Science University, 1-7-1 Kyonancho, Musashino, Tokyo 180-8602, Japan
[f] Faculty of Human Health Science, Hachinohe University, 13-98 Ohaza-Mihono, Hachinohe, Aomori 031-8588, Japan
[g] Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan
[h] Department of Human Ecology, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 1 August 2008
Received in revised form 29 August 2008
Accepted 30 August 2008
Available online 12 September 2008

Keywords:
Cadmium
Neuron
Immunocytochemistry
Proteomics
Synapsin I
MAP2

ABSTRACT

The purpose of the present study was to examine the *in vitro* effects of low-dose cadmium (Cd) on developing cortical cells. The cortical cells removed from fetuses (embryonic day 15) were treated with 10 nM of Cd for 24 h. The effects of Cd on dendritic and synaptic development were immunocytochemically observed with anti-microtubule associated protein-2 (MAP2) and anti-synapsin I antibodies, respectively. Administration of Cd suppressed dendritic as well as synaptic development at 10 nM. By two-dimensional gel electrophoresis and liquid chromatography–tandem mass spectrometric (LC/MS/MS) analysis, we identified three proteins with different expression after Cd-treatment; dihydropyrimidinase-related protein 2 (DRP-2/CRMP-2), 14-3-3-epsillon and calmodulin (CaM). Though the number of identified proteins was small, these proteins are known to be involved in neuronal development. The present study demonstrated the morphological effects as well as affected proteins in Cd-treated cortical cells, providing tools and insights in elucidating mechanisms how low-dose Cd distorts brain development.

1. Introduction

Cadmium (Cd) is an extremely toxic metal commonly found in industrial workplaces, a food contaminant and cigarettes (Piascik et al., 1985; Waalkes et al., 1992; Jarup et al., 1998). Cigarette smoking has been shown to double the Cd intake as compared to non-smokers (Piascik et al., 1985). Furthermore, because Cd is characterized by a long biological half-life (Lind et al., 1997) and a low rate of excretion from the body (Jones and Cherian, 1990), Cd severely damages several organs including kidney (Nordberg, 1984), liver (Koizumi et al., 1996) and testes (Xu et al., 1996; Jones et al., 1997). Due to its high blood–brain barrier permeability (Arvidson and Tjälve, 1986; Choudhuri et al., 1996; Shukla et al., 1996), Cd is also suggested to affect the nervous system. Studies of occupational exposure to Cd have shown the neurobehavioral effects in workers including slowing of visuomotor functioning (Viaene et al., 2000). Increases in complaints about equilibrium alterations and about decreased concentration ability were reported to be dose-dependently associated with urinary Cd levels (Viaene et al., 2000). Parkinsonism (Okuda et al., 1997) and amyotrophic lateral sclerosis (Bar-Sela et al., 2001) were also suggested to have a cause–effect relationship between Cd-exposure.

Besides these suggestive effects of Cd on adults, it has been proposed that Cd can distort brain development. For example, maternal smoking has been linked to altered auditory functioning and reading ability, impulsive behavior and lower IQ score of children (Marlowe et al., 1985a,b; Pihl and Parkes, 1977). WHO/FAO (2004) noted the potential importance of neurodevelopmental toxicity of Cd as following; "Developmental neurobehavioural effects, including decreased locomotor and exploratory activity and certain electrophysiological changes, have been seen in the absence of any overt symptoms of maternal toxicity and appear to be a more sensitive indicator of toxicity".

In experimental animals, neurological pathologic effects of Cd during development including cerebral bleeding and cerebral edema were reported in neonatal mouse (Gabbiani et al., 1967;

* Corresponding author at: Department of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura, Oura, Gunma 374-0193, Japan. Tel.: +81 276 82 9213; fax: +81 276 82 9213.
E-mail address: r-kaneko@toyonet.toyo.ac.jp (R. Ohtani-Kaneko).

0300-483X/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2008.08.021

Webster and Valois, 1981). Cd exposure in infancy caused hyperactivity and increased aggressiveness in juvenile rats (Smith et al., 1982; Holloway and Thor, 1988). Gestational (60 ppm in drinking water or inhalation) and lactational (0.62–2.0 mg/kg subcutaneous injection) exposures to Cd decreased locomoter activity (Barański, 1986; Barański et al., 1983; Lehotzky et al., 1990) and avoidance acquisition in rats (Barański, 1983, 1986). Although all these studies support the idea that Cd even at low doses disturbs brain development, direct neurodevelopmental effects of low-dose Cd and the mechanisms underlying them remain unclear.

Therefore, the purpose of the present study was to examine the effect of low-dose Cd on the development of neurons, especially on dendritic and synaptic development, using the *in vitro* system which we reported in our previous study (Yokosuka et al., 2008). Furthermore, in order to obtain a clue to solve the mechanism of Cd-induced effects, we detected and identified the proteins whose expressions were changed by Cd, using two-dimensional gel electrophoresis (2-DE) and liquid chromatography–tandem mass spectrometric (LC/MS/MS) analysis.

2. Materials and methods

2.1. Procedure of cell culture

Pregnant rats (Japan SLC, Hamamatsu, Japan) were fed on NIH-07PLD laboratory chow (Oriental Yeast, Co. Ltd., Japan), received water ad libitum, and housed in a light and temperature controlled room (lights on 07:00–19:00 h, temperature at 24 °C). Experimental protocols used in this study were in accordance with ethical procedures following the guidelines for the care and use of laboratory animals issued by the Japanese Government and the Japanese Pharmacological Society. Primary cultures of dissociated hypothalamic cells were prepared according to the methods which we previously reported (Yokosuka et al., 2008). In brief, the cerebral cortical tissue was dissected from 30 embryonic day 15 (ED 15; plug = ED 0) rats. After removing the surrounding meninges, the tissue specimens were cut into small pieces. The cortical tissue specimens were pooled and incubated at 37 °C for 15 min in 0.5 U/ml papain (Worthington, USA). After separation from the enzyme solution by sedimentation, the tissue specimens were suspended in a culture medium containing 5% heat-inactivated donor equine serum (Equitech-Bio), 5% heat-inactivated fetal calf serum (Equitech-Bio), and 90% Dulbecco's modified Eagles's medium (DMEM; Gibco BRL) supplemented with 1 mM sodium pyruvate (Sigma), 1.2 mg/ml $NaHCO_3$ (Wako, Japan), 50 units/ml penicillin G (Meiji Seika, Japan) and 25 μg/ml streptomycin sulfate (Meiji Seika, Japan). The cells were then mechanically dissociated by trituration through a plastic pipette. The final cell suspension was plated at a density of 1×10^6 cells/cm^2 in FALCON Culture Flasks and 0.1×10^6 cells/cm^2 in FALCON Culture-Slides (Becton Dickinson) whose bottoms were coated with 0.1% polyethylenimine (Sigma). The cell cultures were maintained in 5% CO_2 and 95% O_2 at 37 °C. After culture in the normal medium for 3 days, the cells were cultured in the medium supplemented with or without 10 nM cadmium chloride for 24 h. We treated cells with 10 nM Cd, because significant suppressions of MAP2- and synapsin I-immunoreactivities were observed at 10^{-6} and 10^{-8} M Cd, when we checked the effects of Cd on them at 10^{-6}, 10^{-8}, 10^{-10} and 10^{-12} M in our preliminary experiments.

3. Morphological analysis

For immunocytochemical staining, cultured cells on culture slides were fixed for 30 min in 4% paraformaldehyde in 100 mM phosphate buffer (PB) after Cd treatment. Following fixation, the cells were incubated with 10 mM phosphate-buffered saline (PBS) containing 0.1% saponin (Sigma) and 10% Block Ace (Dai-Nihon Seiyaku, Japan) at room temperature for 15 min to render the cell membrane permeable and block nonspecific binding. Without washing, cells were incubated with primary antibodies diluted with 10% Block Ace in PBS at 4 °C overnight. Primary antibodies used in this work were as follows: (1) a mouse anti-MAP2 antibody (1:500; Chemicon International) and (2) a rabbit anti-syanpsin I antibody (1:500, Chmicon International). After a brief rinse in PBS, the cells were incubated with biotinylated sheep anti-mouse IgG antibody (1:500, Amersham) diluted with 10% Block Ace in PBS at 4 °C overnight. The cells were then washed in the same manner and incubated for 1 h at room temperature with FITC-conjugated anti-rabbit IgG antibody (1:100, MBL) and Texas Red-labeled streptavidin (1:500, Amersham) diluted with 10% Block Ace in PBS. Finally, after rinsing the cells many times with PBS, coverslips were mounted on the slides using an antifade regent (Fluoro-Gaurd; Bio-Rad). Stained cells were observed under a confocal laser scanning microscope system (LSM 510; Zeiss). Stained cells were optically sectioned at consecutive intervals of 2 μm in the *Z*-axis and the quantification of stained areas was performed as previously described (Yokosuka et al., 2008). Briefly, synapsin I- and MAP2-positive areas were measured with computer-assisted image analysis system (KS-300, Zeiss). Three two-dimensional reconstruction images were analyzed in each well. Each image contained about 20 neurons. The averages were calculated and compared between three Cd-treated wells ($n = 3$) and three control wells ($n = 3$). That is, totally nine images for each cell group were analyzed.

For cell count, cells on culture slides were fixed with 4% paraformaldehyde as described above, stained with Hoechst 33342 (Sigma). Number of cells with normal nuclei was counted in 10 areas of each well. The averages were compared between three control wells ($n = 3$) and three Cd-treated wells ($n = 3$).

4. Sample preparation for electrophoresis

For 2-DE and Western blotting, after medium was removed from culture flasks, cells were rinsed twice in ice–PBS containing phenylmethylsulfonyl fluoride (PMSF) and lysed in lysis buffer (pH 8.5) containing 5 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), 30 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris, Wako Pure Chemical Industries, Japan), 2% 3-[(3-cholamidoproyl) dimethylammonio] propanesulfonate (CHAPS, Dojindo Laboratories, Japan) and 1% dithiothreitol (DTT, Sigma–Aldrich, Germany). The samples were sonicated 5×10 s on ice using an ultra-wave sonicator (Tomy, Japan). Then, the samples were centrifuged at $10{,}000 \times g$ for 30 min at 4 °C. The supernatant was stored at −80 °C until gel electrophoresis.

5. Two-dimensional gel electrophoresis

Each 100 μg aliquot of either control or Cd-treated protein samples was loaded onto a 24 cm Immobiline Dry-Strips covering the ranges of pH 4.0-7.0 (GE Healthcare) for isoelectric focusing (IEF) using the IPGphor isoelectric focusing system (GE Healthcare). After IEF, the strips were equilibrated in the first equilibration solution (1.5 M Tris–HCl, pH 8.8; 6 M urea, 30% glycerol) (Wako Pure Chemical Industries), 2% sodium dodecyl sulfate (SDS, Wako Pure Chemical Industries), 10 mg/ml DTT (Wako Pure Chemicals) and were then equilibrated in the second equilibration solution (the SDS equilibration buffer containing 25 mg/ml iodoacetamide (Wako Pure Chemical Industries)). The equilibrated strips were placed on top of 9–18% SDS-polyacrylamide gradient gels and sealed with a solution of 1% (w/v) agarose (Takara Bio, Japan). The second electrophoresis was carried out using the Ettan DALTsix system (GE Healthcare).

After electrophoresis, the gels were stained using SYPRO Ruby stain solution (Molecular Probes, Eugnene, OR, USA), de-stained in 10% ethanol and 6% acetic acid, according to the protocol recommended by the manufacturer. The gel was then scanned at an excitation wavelength of 457 nm and at an emission wavelength of 610 nm. Changed (decreased or increased) spots by cadmium treatment were selected. In addition, two more gels, one for control and the other for Cd-treated samples, were stained with silver staining kit (GE Healthcare). The gels were dried and stored at 4 °C until MS analysis.