Free Radical Biology & Medicine 45 (2008) 1035–1044

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Cadmium activates the mitogen-activated protein kinase (MAPK) pathway via induction of reactive oxygen species and inhibition of protein phosphatases 2A and 5

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ARTICLE INFO

Article history:
Received 2 June 2008
Revised 12 July 2008
Accepted 16 July 2008
Available online 26 July 2008

Keywords:
Cadmium
Apoptosis
Reactive oxygen species
Protein phosphatase 2A
Protein phosphatase 5
c-Jun N-terminal kinase
Extracellular signal-regulated kinase 1/2

ABSTRACT

Cadmium (Cd), a highly toxic environmental pollutant, induces neurodegenerative diseases. Recently we have demonstrated that Cd may induce neuronal apoptosis in part through activation of c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (Erk1/2) pathways. However, the underlying mechanism remains enigmatic. Here we show that Cd induced generation of reactive oxygen species (ROS), leading to apoptosis of PC12 and SH-SY5Y cells. Pretreatment with *N*-acetyl-L-cysteine (NAC) scavenged Cd-induced ROS, and prevented cell death, suggesting that Cd-induced apoptosis is attributed to its induction of ROS. Furthermore, we found that Cd-induced ROS inhibited serine/threonine protein phosphatases 2A (PP2A) and 5 (PP5), leading to activation of Erk1/2 and JNK, which was abrogated by NAC. Overexpression of PP2A or PP5 partially prevented Cd-induced activation of Erk1/2 and JNK, as well as cell death. Cd-induced ROS was also linked to the activation of caspase-3. Pretreatment with inhibitors of JNK (SP600125) and Erk1/2 (U0126) partially blocked Cd-induced cleavage of caspase-3 and prevented cell death. However, zVAD-fmk, a pan caspase inhibitor, only partially prevented Cd-induced apoptosis. The results indicate that Cd induction of ROS inhibits PP2A and PP5, leading to activation of JNK and Erk1/2 pathways, and consequently resulting in caspase-dependent and -independent apoptosis of neuronal cells. The findings strongly suggest that the inhibitors of JNK, Erk1/2, or antioxidants may be exploited for prevention of Cd-induced neurodegenerative diseases.

Introduction

Cadmium (Cd), a highly toxic heavy metal, is mainly released from the smelting, burning of fossil fuels and municipal wastes, refining of metals, and cigarette smoking, resulting in the pollution of water, air, and soil. Exposure of human or animals to a Cd-contaminated environment or food chain causes accumulation of Cd in many organs, including kidney [1,2], liver [3], lung [4,5], testis, bone, etc. [6,7], and thereby contributes to carcinogenesis, immunodepression, and neurodegeneration [8,9]. Clinical data show that exposure to Cd induces neurological disorders, such as learning disabilities and hyperactivity in children [10,11], olfactory dysfunction and neurobehavioral defects in attention, psychomotor speed, and memory in workers [7,12]. Therefore, Cd intoxication has been considered and studied as a possible etiological factor of neurodegenerative diseases.

Studies have demonstrated that the toxicity of Cd is related to its induction of oxidative stress, e.g., reactive oxygen species (ROS), in various types of cells [7,12–14]. Elevated levels of ROS may cause increased permeability of the blood–brain barrier, tubulin alterations, and perturbation in synaptic transmission [7]. Oxidative stress is a prominent feature of many neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Cd-induced oxidative stress is closely associated with PD and AD [13,15–18]. Accumulating data further show that under pathological conditions, excessive amounts of ROS induced by Cd can modify proteins, lipids, and DNA, alter their functions, and activate related signaling pathways, thereby resulting in apoptosis of neuronal cells [12,14,16,19–21].

There is growing evidence that members of the mitogen-activated protein kinase (MAPK) family may play a critical role in neuronal apoptosis [22]. MAPKs comprise a highly conserved cascade of serine/

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ASK1, apoptosis signal-regulating kinase 1; Cd, cadmium; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle medium; Erk1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKP1, MAP phosphatase 1; NAC, *N*-acetyl-L-cysteine; OD, optical density; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PDL, poly-D-lysine; PI, propidium iodide; PD, Parkinson's disease; PP2A, protein phosphatase 2A; PP5, protein phosphatase 5; ROS, reactive oxygen species; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone.

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doi:10.1016/j.freeradbiomed.2008.07.011

threonine kinases connecting cell surface receptors to regulatory targets in response to various stimuli [16,22,23]. Mammals express at least three distinct groups of MAPKs, including extracellular signal-regulated protein kinase 1/2 (Erk1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK, which have been well characterized. In neuronal cells, Erk1/2 is primarily activated by growth factors, and is involved in cellular proliferation, differentiation, and development, whereas JNK and p38 signaling cascades are preferentially activated by environmental stress and inflammatory cytokines, and have been shown to promote neuronal cell death [24]. Phosphorylation of MAPKs is balanced by specific MAPK kinases and phosphatases. MAPK kinase 1/2, MAPK kinase 3/6, and MAPK 4/7 are the major kinases to phosphorylate Erk1/2, p38, and JNK, respectively, though there exist cross talks [22]. MAP phosphatase 1 (MKP1) and serine/threonine protein phosphatase 2A (PP2A) are the major phosphatases that negatively regulate phosphorylation of Erk1/2, JNK, and p38 [25,26]. Protein phosphatase 5 (PP5) has been identified as a negative regulator of JNK cascade, involved in stress responses [27,28]. PP2A is a heterotrimeric holoenzyme composed of a catalytic subunit (PP2Ac), an A subunit (also termed PR65), and a number of B subunits, including B (PR55), B′ (PR61), B″ (PR72), and B‴ (PR93/PR110) [29]. The phosphatase activity of PP2Ac or PP5 is modulated by their associations with PP2A-A and -B regulatory subunits [27]. Furthermore, PP2A activity is also mediated by the phosphorylation or methylation of PP2Ac [30,31]. Recently, we have found that all three MAPK members can be activated by Cd in neuronal cells, and identified that Cd-induced neuronal apoptosis is partially associated with activation of JNK, Erk1/2, but not by p38 signaling [32]. However, it is less clear how Cd activates MAPKs signaling pathways in the neuronal cells.

Activation of JNK and p38 MAPK by ROS has been described [24]. This prompted us to study whether Cd activates MAPKs signaling via its induction of ROS in neuronal cells. Since fully developed neurons are permanently amitotic (not dividable), transformed PC12 and SH-SY5Y cells, two established cell lines derived from rat adrenal medulla and human sympathetic neurons, respectively, are widely used as in vitro neuronal models, Here we show that Cd activation of MAPKs is indeed related to its induction of ROS generation in neuronal (PC12 and SH-SY5Y) cells. This is supported by the findings that Cd induced generation of ROS; *N*-acetyl-L-cysteine (NAC), an antioxidant and ROS scavenger, effectively blocked Cd-induced activation of Erk1/2, JNK, and p38 signaling network, and prevented Cd-induced cell death. We also found that the activation of MAPKs is associated with inhibition of PP2A and PP5 by Cd-induced ROS. Furthermore, we observed that Cd-induced ROS activated caspase-3, which could be blocked by NAC. Treatment with zVAD-fmk, a pan caspase inhibitor, only partially protected against Cd-induced cell death, implying that caspase-dependent and -independent apoptotic mechanisms are involved. Pretreatment with MAPK inhibitors (SP600125 and U0126) partially inhibited Cd-induced cleavage of caspase-3 and apoptosis, suggesting that those inhibitors, plus antioxidant (e.g., NAC), may be exploited for prevention of Cd-induced neurodegenerative diseases.

Materials and methods

Materials

Cadmium chloride (Sigma, St. Louis, MO) was dissolved in sterile distilled water to prepare the stock solutions (0–120mM), aliquoted, and stored at room temperature. Dulbecco's modified Eagle medium (DMEM) was purchased from Mediatech (Herndon, VA). Horse serum and fetal bovine serum (FBS) were supplied by Hyclone (Logan, UT), whereas 0.05% Trypsin-EDTA was from Invitrogen (Grand Island, NY). Enhanced chemiluminescence solution was from Pierce (Rockford, IL). CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit was from Promega (Madison, WI). The MAPK inhibitors, SB203580, U0126, and SP600125, were obtained from LC Laboratories (Woburn, MA). The caspase inhibitor, z-Val-Ala-Asp-CH2F (zVAD-fmk) was purchased from ALEXIS Biochemicals Corporation (San Diego, CA). The following antibodies were used: ASK1, phospho-ASK1 (Thr845), MKK4, phospho-MKK4 (Ser257/Thr261), phospho-Erk1/2 (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA), PP2ACα, PP5 (BD Biosciences, San Jose, CA), PP2A-A subunit, PP2A-B subunit (Upstate, Lake Placid, NY), JNK1, phospho-JNK (Thr183/Tyr185), c-Jun, phospho-c-Jun (Ser63), Erk2, p38, phospho-p38 (Thr180/Tyr182), MEK1/2, phospho-MEK1/2, MEK3/6, phospho-MEK3/6, demethylated-PP2A, MKP1, FLAG, HA (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-PP2A (Epitomics, Burlingame, CA), and β-tubulin (Sigma). Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Biosciences. 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) and propidium iodide (PI) were from MP Biomedicals Inc. (Solon, OH), and Alfa Aesar (Ward Hill, MA), respectively. *N*-Acetyl-L-cysteine, poly-D-lysine (PDL), 4′,6-diamidino-2-phenylindole (DAPI), and all the other chemicals were purchased from Sigma.

Cell culture

Rat pheochromocytoma (PC12) and human neuroblastoma (SH-SY5Y) cell lines were from American Type Culture Collection (ATCC) (Manassas, VA). PC12 cells were grown in antibiotic-free DMEM supplemented with 10% horse serum and 5% FBS, whereas SH-SY5Y cells were grown in antibiotic-free DMEM supplemented with 10% FBS. Cells were maintained in a humid incubator (37°C, 5% CO_2).

Recombinant adenoviral constructs and infection of cells

Plasmids encoding N-terminal FLAG-tagged wild-type rat PP2Aα catalytic subunit (PP2Ac-α) and hemagglutinin (HA)-tagged wild-type human PP5 [27,28] were gifts from Dr. Hitoshi Nakagama (Hokkaido University, Sapporo, Japan) and Dr. Hidenori Ichijo (University of Tokyo, Tokyo, Japan), respectively. The recombinant adenoviruses encoding FLAG-tagged PP2Ac-α (Ad-PP2A), HA-tagged PP5 (Ad-PP5), and the control virus encoding the green fluorescence protein (GFP) (Ad-GFP) were generated using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA), following the manufacture's instruction. The viruses were amplified and titrated as described [33]. For experiments, PC12 and SH-SY5Y cells were grown in the growth medium, and infected with the individual adenovirus for 24h at 5 of multiplicity of infection (MOI = 5). Subsequently, cells were used for experiments. Ad-GFP served as a control. Expression of FLAG-tagged PP2Ac-α and HA-tagged PP5 was determined by Western blot with antibodies to FLAG and HA (Santa Cruz Biotechnology), respectively.

Analysis for generation of ROS

The production of ROS was measured by detecting the fluorescent intensity of oxidant-sensitive probe CM-H_2DCFDA, which is a stable nonfluorescent molecule that passively diffuses into cells, where the acetate can be cleaved by intracellular esterases to produce a polar diol that is well retained within the cells. PC12 cells were seeded at a density of 1×10^4 cells/well in 96-well plate, precoated with PDL (0.2μg/ml). The next day, cells were loaded with CM-H_2DCFDA as per the manufacturer's protocol and incubated in the presence of various concentrations of Cd (0–120μM) for 24h or 20μM Cd for different times (0–24h) with 6 replicates of each treatment. In some cases, cells were preincubated with NAC (5mM) for 1h, and then treated with/without $CdCl_2$ (10 and 20μM) for 24h, followed by loading with CM-H_2DCFDA for 40min. For experiments with inhibitors, including the MAPK inhibitors, SB203580, U0126, and SP600125 as well as the caspase inhibitor zVAD-fmk, cells were preincubated with each inhibitor for