Toxicology and Applied Pharmacology 254 (2011) 65–71

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Effects of manganese on tyrosine hydroxylase (TH) activity and TH-phosphorylation in a dopaminergic neural cell line

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ARTICLE INFO

Article history:
Received 30 September 2008
Revised 17 March 2010
Accepted 17 March 2010
Available online 7 February 2011

Keywords:
Manganese
Neurotoxicity
Dopamine synthesis
Protein kinase C
Phosphorylation
Parkinson's disease

ABSTRACT

Manganese (Mn) exposure causes manganism, a neurological disorder similar to Parkinson's disease. However, the cellular mechanism by which Mn impairs the dopaminergic neurotransmitter system remains unclear. We previously demonstrated that caspase-3-dependent proteolytic activation of protein kinase C delta (PKCδ) plays a key role in Mn-induced apoptotic cell death in dopaminergic neurons. Recently, we showed that PKCδ negatively regulates tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, by enhancing protein phosphatase-2A activity in dopaminergic neurons. Here, we report that Mn exposure can affect the enzymatic activity of TH, the rate-limiting enzyme in dopamine synthesis, by activating PKCδ–PP2A signaling pathway in a dopaminergic cell model. Low dose Mn (3–10 μM) exposure to differentiated mesencephalic dopaminergic neuronal cells for 3 h induced a significant increase in TH activity and phosphorylation of TH-Ser40. The PKCδ specific inhibitor rottlerin did not prevent Mn-induced TH activity or TH-Ser40 phosphorylation. On the contrary, chronic exposure to 0.1–1 μM Mn for 24 h induced a dose-dependent decrease in TH activity. Interestingly, chronic Mn treatment significantly increased PKCδ kinase activity and protein phosphatase 2A (PP2A) enzyme activity. Treatment with the PKCδ inhibitor rottlerin almost completely prevented chronic Mn-induced reduction in TH activity, as well as increased PP2A activity. Neither acute nor chronic Mn exposures induced any cytotoxic cell death or altered TH protein levels. Collectively, these results demonstrate that low dose Mn exposure impairs TH activity in dopaminergic cells through activation of PKCδ and PP2A activity.

Introduction

Chronic exposure to manganese (Mn) has been shown to cause a Parkinson's-like syndrome known as manganism. Increased incidences of manganism have been observed among miners and industrial welders, as well as farmers exposed to Mn-based pesticides such as the fungicides Maneb (Mn ethylene-bis-dithiocarbamate) and Mancozeb [[2-[(dithiocarboxy)amino]ethyl]-[carbamodithioato(2-)-κS,κS′]-zinc] (Roth and Garrick, 2003; Dobson et al., 2004). Adverse neurological effects of Mn also occurred in people who drank water containing high levels of Mn in many countries and in abusers who used the Mn-containing compound Bazooka, a cocaine-based drug (Ensing, 1985). Exposures to infants and young children through infant food formulations have also been documented (Aschner and Aschner, 2005; Erikson et al., 2007). The recent introduction of the Mn-containing fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT) to gasoline has raised concerns over potential chronic exposures to Mn.

Several lines of evidence suggest that exposure to Mn or Mn-containing compounds induces a variety of cellular changes including glutathione and dopamine depletion, increased oxidative stress, and impairment of energy metabolism and antioxidant systems (Kitazawa et al., 2002; Roth and Garrick, 2003; Dobson et al., 2004; Hirata et al., 2004; Olanow, 2004). Studies have also shown the role of Mn intoxication in astrocyte-derived injury to striatal–pallidal interneurons (Liu et al., 2006). The nigrostriatal system, including the globus pallidus and substantia nigra, is the primary target region of Mn neurotoxicity. Recently, we developed an in vitro model of dopaminergic neurotoxicity, namely N27 cells derived from the mesencephalon, a brain region directly affected by Parkinson's disease. The N27 cells also represent a homogenous population of tyrosine hydroxylase-positive cells with functional characteristics similar to dopaminergic neurons, including dopamine synthesis and oxidative stress cellular signaling (Kaul et al., 2003; Anantharam et al., 2004). We recently observed that caspase-3-mediated proteolytic activation of the proapoptotic kinase PKCδ plays a role in Mn-induced apoptosis in N27 mesencephalic dopaminergic cells (Latchoumycandane et al., 2005).

Depletion of dopamine in nigrostriatal neurons has been documented during Mn exposure (Goldstein and Lieberman, 1992; Aschner et al., 2006; Liu et al., 2006; Perl and Olanow, 2007; Erikson

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doi:10.1016/j.taap.2010.03.023

et al., 2008; Guilarte et al., 2008). Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the biosynthesis of catecholamines and catalyzes the first step of a biochemical synthetic pathway in which L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA). Phosphorylation and dephosphorylation of TH represent important post-translational regulatory mechanisms of the enzymatic activity that in turn regulates the amount of catecholamine synthesized in a dopaminergic system (Goldstein and Lieberman, 1992; Dunkley et al., 2004; Fujisawa and Okuno, 2005). TH-Ser40 is a major residue that positively regulates the TH activity in vivo (Campbell et al., 1986; Wu et al., 1992). In addition to TH-Ser40 phosphorylation, TH activity can be regulated by phosphorylation of serine residues at positions 8, 19, 31 and 40, resulting in enhanced dopamine synthesis (Lee et al., 1989; Haycock, 1990; Haycock et al., 1992).

The phosphorylation state of TH can also be regulated by dephosphorylation reactions mediated by phosphatases. Protein phosphatase 2A (PP2A) has been demonstrated to be the major serine/threonine phosphatase that dephosphorylates TH, resulting in reduced TH activity (Haavik et al., 1989). Recently, we reported a novel functional interaction between PKCδ and TH, in which PKCδ negatively regulates TH activity and dopamine synthesis by enhancing PP2A activity in dopaminergic neurons (Zhang et al., 2007). Herein, we examined whether Mn exposure alters TH activity and dopamine synthesis by modulating PKCδ-PP2A signaling.

Methods

Chemicals. Manganese chloride (MnCl2; 99%), rottlerin, NSD-1015, dibutyryl cAMP, protease cocktail, and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO); phosphoTH-Ser40 antibody was purchased from Chemicon (Temecula, CA); rabbit PKCδ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Anti-rabbit and anti-mouse secondary antibodies and the ECL chemiluminescence kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). RPMI, fetal bovine serum, L-glutamine, penicillin, and streptomycin were purchased from Invitrogen (Gaithersburg, MD).

Cell culture models. Differentiated N27 cells were cultured, as described in our earlier publications (Adams et al., 1996; Anantharam et al., 2004). Cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U of penicillin, and 50 μg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. N27 cells were differentiated with 2 mM dibutyryl cAMP for 3–5 days and then used for experiments described below.

Treatment paradigm. Differentiated N27 dopaminergic cells were exposed to 0.1–10 μM $MnCl_2$ for the duration of the experiment. For measurement of TH activity, cells were exposed to 2 mM NSD-1015 for 1 h prior to $MnCl_2$ treatment. Untreated cells were used as control samples. In inhibitor studies, 3 μM rottlerin was used for PKCδ inhibition. We derived the concentrations of rottlerin used in this study from previously published literature. Rottlerin inhibits PKCδ activity with a Ki of 3–6 μM, whereas rottlerin inhibits PKCδ, β, γ, ε, and λ with Ki values at least 5–10 times higher (Gschwendt, 1999; Kanthasamy et al., 2003; Reyland, 2007). In our previous study, we showed 3–10 μM rottlerin attenuated kinase activity to a greater extent (Anantharam et al., 2002). For this study, 3 μM rottlerin was used, which was lower than the Ki values of other PKC isoforms.

Cytotoxicity assay. Cytotoxicity measurements were performed using Sytox Green assay, as described previously (Anantharam et al., 2007). Membrane-impermeable DNA dye Sytox Green readily enters dying cells, resulting in increased fluorescence. The intensity of fluorescence is directly proportional to the amount of dead cells. After growing N27 cells in 24-well plates for 24 h, cells were immediately exposed to 300 μM MPP+ in the presence of NADPH oxidase inhibitors (100–1000 μM AEBSF, 100–1000 μM apocynin, and 3–30 μM DPI) in a 1 μM Sytox-containing growth media. After 24 h, cytotoxic cell death was quantified by measuring DNA-bound Sytox Green using a Gemini fluorescence microplate reader (Ex 485 nm and Em 538 nm; Molecular Devices Corporation). Fluorescent images of Sytox-positive cells were taken after 24 h exposure with a Nikon TE2000 microscope, and pictures were captured with a SPOT digital camera.

Tyrosine hydroxylase activity. TH enzyme activity was measured by the modified method of Hayashi et al. (1988), in which DOPA levels are quantified as an index of TH activity after inhibition of DOPA decarboxylase with the decarboxylase inhibitor NSD-1015. Briefly, cells were incubated with Krebs–HEPES buffer (pH 7.4) containing 2 mM NSD-1015 at 37 °C for 1 h and then subjected to the treatment paradigm, as described earlier. After treatment, cells were collected and resuspended in antioxidant solution, sonicated, and centrifuged. DOPA levels in the supernatants were measured by high-performance liquid chromatography with electrochemical detection (HPLC-EC). Samples were prepared as described previously (Kitazawa et al., 2001; Sun et al., 2006). Neurotransmitters were extracted from samples using 0.1 M perchloric acid containing 0.05% Na_2EDTA and 0.1% $Na_2S_2O_5$. The extracts were filtered in 0.22 micron spin tubes and 20 μl of the samples were loaded for analysis. DOPA was separated isocratically by a reversed-phase column with a flow rate of 0.7 ml/min. An HPLC system (ESA Inc., Bedford, MA) with an ESA automatic sampler (model 542) was used for these experiments. The electrochemical detection system consisted of an ESA Coulochem Model 5100A with a microanalysis cell model 5014A and a guard cell model 5020 (ESA Inc., Bedford, MA). The peak areas of standard DOPA were compared with sample areas. The DOPA levels in the samples were measured and expressed as picogram per microgram protein, and retention times for DOPA were 2.5–4 min.

PP2A assay. To determine PP2A activity, the serine/threonine phosphatase assay kit was used (Promega, Madison, WI). For PP2A activity measurement, N27 cells were homogenized in lysis buffer (25 mM Tris–HCl, 10 mM β-mercaptoethanol, 2 mM EDTA, protease inhibitor) supplied with the kit. After centrifugation, the supernatants were used for the assay. PP2A activity was determined by the amount of free phosphate generated in a reaction and measured by the absorbance of a molybdate:malachite green: phosphate complex at 600 nm using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). The effective range for the detection of phosphate released in this assay is 100–4000 pmol of phosphate.

Western blotting. Cell lysates containing equal amounts of protein were loaded in each lane and separated on a 10–12% SDS–PAGE gel, as described previously (Kaul et al., 2003). After the separation, proteins were transferred to nitrocellulose membranes and nonspecific binding sites were blocked by treating with 5% nonfat dry milk powder. The membranes were then treated with primary antibodies directed against PKCδ (rabbit polyclonal or mouse monoclonal; 1:2000 dilution) and phospho TH-Ser40 (rabbit polyclonal; 1:1000). The primary antibody treatments were followed by treatment with secondary HRP-conjugated anti-rabbit IgG (1:2000) for 1 h at RT. Secondary antibody-bound proteins were detected using Amersham's ECL chemiluminescence kit. To confirm equal protein loading, blots were reprobed with β-actin antibody (1:5000 dilution). Western blot images were captured with a Kodak 2000 MM imaging system and data were analyzed using 1D Kodak imaging analysis software.

Data analysis. Data analysis was performed using Prism 4.0 software (GraphPad Software, San Diego, CA). Data were analyzed