Published in final edited form as:

Toxicol Appl Pharmacol. 2011 July 15; 254(2): 65–71. doi:10.1016/j.taap.2010.03.023.

Effects of Manganese on Tyrosine Hydroxylase (TH) Activity and TH-phosphorylation in a Dopaminergic Neural Cell Line

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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 (PKC δ) plays a key role in Mn-induced apoptotic cell death in dopaminergic neurons. Recently, we showed that PKC δ 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 PKC δ - PP2A signaling pathway in a dopaminergic cell model. Low dose Mn (3–10 μ M) exposure to differentiated mesencephalic dopaminergic neuronal cells for 3 h induced a significant increase in TH activity and phosphorylation of TH-Ser40. The PKC δ specific inhibitor rottlerin did not prevent Mn-induced TH activity or TH-Ser40 phosphorylation. On the contrary, chronic exposure to 0.1–1 μ M Mn for 24 h induced a dose-dependent decrease in TH activity. Interestingly, chronic Mn treatment significantly increased PKC δ kinase activity and protein phosphatase 2A (PP2A) enzyme activity. Treatment with the PKC δ 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 PKC δ and PP2A activity.

Keywords

Manganese; Neurotoxicity; Dopamine synthesis; Protein Kinase C; phosphorylation; Parkinson's disease

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-

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[(dithiocarboxy)amino]ethyl]-carbamodithioato(2-)- $\kappa S, \kappa S'$]-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.*, 2003a; Anantharam *et al.*, 2004). We recently observed that caspase-3-mediated proteolytic activation of the proapoptotic kinase PKC δ 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 *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 PKC δ and TH, in which PKC δ 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 PKC δ -PP2A signaling.

Methods

Chemicals

Manganese chloride (MnCl₂, 99%), rottlerin, NSD-1015, dibutyl 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 PKC δ 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 units of penicillin, and 50 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ 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 μ M MnCl₂ for the duration of the experiment. For measurement of TH activity, cells were exposed to 2 mM NSD-1015 for 1 h prior to MnCl₂ treatment. Untreated cells were used as control samples. In inhibitor studies, 3 μ M rottlerin was used for PKC δ inhibition. We derived the concentrations of rottlerin used in this study from previously published literature. Rottlerin inhibits PKC δ activity with a Ki of 3–6 μ M, whereas rottlerin inhibits PKC δ , 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 μ M rottlerin attenuated kinase activity to a greater extent (Anantharam *et al.*, 2002). For this study, 3 μ M 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 μ M MPP⁺ in the presence of NADPH oxidase inhibitors (100–1000 μ M AEBSF, 100–1000 μ M apocynin and 3–30 μ M DPI) in a 1 μ M 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.*, in which DOPA levels are quantified as an index of TH activity after inhibition of DOPA decarboxylase with the decarboxylase inhibitor NSD-1015 (Hayashi *et al.*, 1988). 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% Na₂ EDTA and 0.1%

Na₂S₂O₅. 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 pg/µg 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 100–4,000 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.*, 2003b). 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 using one-way ANOVA, and then Bonferroni's post-test was performed to compare all treatment groups. Differences with $p < 0.05$ were considered significant.

Results

Acute Mn exposure induces increases in TH activity

Recently we demonstrated that protein kinase C delta (PKCδ) negatively regulates tyrosine hydroxylase (TH) activity and dopamine synthesis by enhancing protein phosphatase 2A activity in dopaminergic neurons, both in cell culture and in animal brain (Zhang *et al.* 2007). PKCδ mediates apoptotic cell death in undifferentiated N27 dopaminergic neuronal cells following a high concentration of Mn (300 µM) for 24 h (Latchoumycandane *et al.* 2005). In this study, we examined whether Mn at non-toxic doses had any effect on TH activity. Differentiated N27 cells were exposed to 3 µM and 10 µM Mn, and after 3 h, cells were harvested, lysed, and measured for TH activity. As shown in Fig. 1A, both 3 µM and 10 µM Mn significantly increased TH activity as compared to untreated control cells. The increase was about 77% over control level (Fig 1B). To determine if PKCδ mediates Mn-

induced increases in TH activity, N27 cells were pretreated with 3 μ M rottlerin for 30 min prior to Mn treatment. The results show that Mn-induced increases in TH activity were unaffected by rottlerin treatment (Fig. 1A).

Acute Mn exposure increases TH phosphorylation in dopaminergic cells

We previously demonstrated that PKC δ colocalizes with TH and also negatively regulates TH-Ser40 phosphorylation (Zhang *et al.* 2007). Since acute Mn exposure resulted in enhanced TH activity, we examined whether acute Mn treatment has any effect on the phosphorylation status of TH at Ser40. Differentiated N27 cells were exposed to 3 μ M and 10 μ M Mn for 3 h and the cell lysate was subjected to TH-Ser40 phospho-immunoblot analysis. As shown in Fig. 2, Mn treatment induced dose-dependent increases in the levels of TH-Ser40 phosphorylation. Densitometry analysis of the 60 kDa TH-Ser40 band in Fig. 2A revealed an almost twofold increase in phosphorylation in 10 μ M Mn-treated cells compared to untreated control cells. A 43 kDa β -actin band was used for confirming equal protein loading of samples in each lane. These results demonstrate that acute Mn exposure results in enhanced TH phosphorylation.

Acute Mn exposure is not toxic to differentiated N27 cells

Next we determined if a 3 h exposure to 3–10 μ M Mn induces any cytotoxicity in differentiated N27 cells. Cytotoxicity was measured using the Sytox Green fluorescence assay. An increase in the number of Sytox-positive green cells indicates an increase in cell death because the Sytox Green dye permeates compromised cell membranes to stain nuclear chromatin. Quantitative analysis of Sytox fluorescence using a fluorescence plate reader further confirmed that acute Mn exposure (3–10 μ M) does not produce any significant cytotoxic response in differentiated N27 cells (Fig. 3). H₂O₂-treated N27 cells, used as a positive control, showed a fivefold increase in Sytox fluorescence (Fig. 3). These results suggest that the Mn concentration used in acute experiments is not toxic to the cells.

Chronic Mn exposure decreases TH activity in N27 cells

In the next set of experiments, we examined whether chronic Mn treatment also modulates TH activity. N27 cells were exposed to 0.1–1 μ M Mn for 24 h, and TH activity was measured in the cell lysates. As shown in Fig. 4A, Mn induced a dose-dependent decrease in TH activity. Comparison with untreated controls revealed that TH activity was decreased to 70%, 61% and 59% in 0.1, 0.3 and 1 μ M Mn-treated cells, respectively (Fig. 4B). Thus, unlike acute exposure, chronic Mn treatment produced a decrease in TH activity. Western blot analysis of TH-Ser40 phosphorylation revealed a marginal decrease in TH-Ser40 phosphorylation in 1 μ M Mn-treated samples (Fig. 4C), however the decrease was not statistically significant. The lower concentrations of Mn did not significantly alter TH-Ser40 phosphorylation as compared to untreated samples (data not shown).

Chronic Mn exposure is not toxic to differentiated N27 cells

Next we examined if a 24 h exposure to 0.1–1 μ M Mn induces cytotoxicity in differentiated N27 cells. Cytotoxicity was measured using the Sytox Green fluorescence assay. As shown in Fig. 5, quantitative analysis of Sytox fluorescence using a fluorescence plate reader revealed that differentiated N27 cells were unaffected by chronic Mn treatment (0.1–1 μ M for 24 h). H₂O₂-treated N27 cells were again used as a positive control and a 100 μ M H₂O₂ treatment for 3 h induced a greater than fivefold increase in Sytox fluorescence (Fig. 5). These results suggest that the Mn concentration used in chronic experiments is not toxic to the cells.

Rottlerin blocks Mn-induced changes in TH and PKC δ activities

To determine whether PKC δ plays any role in the chronic Mn-induced decreases in TH activity as shown in Fig. 4, differentiated N27 cells were cotreated with the PKC δ inhibitor rottlerin (3 μ M). As shown in Fig. 6A, rottlerin cotreatment completely restored the TH activity in Mn-treated cells to the level of the untreated control cells. These data suggest that PKC δ plays a role in reduced TH activity during chronic Mn exposure in dopaminergic neuronal cells.

Since rottlerin treatment prevented Mn-induced decreases in TH activity, we examined if chronic Mn treatment has any effect on PKC δ activity. Differentiated N27 cells were exposed to 1 μ M Mn in the presence or absence of 3 μ M rottlerin and the cell lysates were used in the kinase assay. PKC δ was immunoprecipitated from the cell lysates and the kinase assay was performed by measuring PKC δ phosphorylation of histone H1 using [32 P]-ATP, as described previously (Anantharam et al., 2002). As shown in Fig. 6B, a 24-h exposure to 1 μ M Mn resulted in a 40% increase in histone phosphorylation compared to untreated control cells. Importantly, rottlerin cotreatment completely suppressed 1 μ M Mn-induced increases in kinase activity. Pure recombinant PKC δ protein was used as a positive control. Together, these data imply that chronic exposure to low doses of Mn induces increases in PKC δ activity and that rottlerin at 3 μ M effectively suppresses Mn-induced PKC δ activation.

PKC δ mediates Mn-induced increases in PP2A activity in dopaminergic cells

We previously demonstrated that PKC δ negatively regulates TH activity by enhancing protein phosphatase 2A (PP2A) activity in dopaminergic neurons (Zhang *et al.* 2007). Since chronic Mn treatment induced increases in PKC δ activity and decreased TH activity, we examined whether chronic Mn treatment also increases PP2A activity. As shown Fig. 7, PP2A enzyme activity increased by almost 50% in differentiated N27 cells exposed to 1 μ M Mn for 24 h. Furthermore, 3 μ M rottlerin significantly blocked Mn-induced increases in PP2A activity, suggesting that PKC δ may be activating PP2A to reduce TH activity.

Discussion

In the present study, we demonstrate that short-term and long-term low-dose Mn exposures have opposing effects on TH activity. While acute exposure to low μ M Mn concentrations (1–10 μ M for 3 h) increased TH activity, chronic exposure to 0.1–1 μ M Mn over 24 h had an opposite effect (decreased TH activity) in differentiated dopaminergic neuronal cells. Chronic Mn exposure also caused PKC δ activation and increased PP2A activity, and these increases were blocked by the PKC δ inhibitor rottlerin. Importantly, inhibition of PKC δ reversed the chronic Mn-induced decrease in TH activity. The observed biochemical changes are independent of cytotoxicity because the doses of Mn used in the study did not induce any cytotoxicity. To our knowledge this is the first report showing that manganese regulates TH activity in dopaminergic neuronal cells via PKC δ and PP2A.

The nigrostriatal system, including the globus pallidus and substantia nigra, is the primary target of Mn (Baek *et al.*, 2003). Accumulation of manganese in pallidal regions of the basal ganglia has been well described as ‘manganese hyperintensity signals’ by T1-weighted MRI imaging procedures (Krieger *et al.*, 1995). Environmental Mn exposure range from moderate, as occurs via drinking water and food sources, to high, as found in occupational and industrial settings such as mining, welding, and steel manufacturing (Woolf *et al.*, 2002; Roth and Garrick, 2003; Dobson *et al.*, 2004; Olanow, 2004). The mean Mn level in the human brain is 0.261 μ g/g, with wet weights of 6.31, 0.34, and 58.5 ng/mg in the putamen, substantia nigra, and neuromelanin, respectively (Zecca *et al.*, 2004). Depending on the level

of exposure, Mn levels can increase from 10 to 200-fold (Hauser *et al.*, 1996; Lucchini *et al.*, 1999; Woolf *et al.*, 2002; McKinney *et al.*, 2004). Higher concentrations (>100 μ M) of Mn are generally required to observe neurotoxicity in cell cultures (Roth and Garrick, 2003; Hirata *et al.*, 2004; Stredrick *et al.*, 2004; Latchoumycandane *et al.*, 2005), but we found that differentiated mesencephalic N27 dopaminergic cells are very sensitive to low doses of Mn, suggesting the utility of this model for studying neurochemical mechanisms underlying manganese neurotoxicity.

We previously showed that inorganic Mn (>100 μ M) and an organic Mn compound, methylcyclopentadienyl manganese tricarbonyl (MMT, >300 μ M), induce ROS generation, mitochondrial dysfunction and apoptotic cell death in two different dopaminergic cell lines: PC12 and undifferentiated N27 cells. (Anantharam *et al.*, 2002; Latchoumycandane *et al.*, 2005). We had also demonstrated that high-doses of Mn (100–300 μ M) induced caspase-3-dependent proteolytic cleavage of PKC δ in undifferentiated N27 cells (Latchoumycandane *et al.* 2005). We recently showed that N27 cells express DMT1 and other metal transporter proteins that may play a role in Mn neurotoxicity. (Afeseh Ngwa *et al.*, 2009)

TH is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to dihydroxyphenylalanine (DOPA), which is subsequently converted to dopamine, a key neurotransmitter that controls extrapyramidal motor function in the basal ganglia. TH activity can be regulated post-translationally 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). Recently, we demonstrated that PKC δ colocalizes and physically interacts with TH and PP2A, and that PKC δ phosphorylates PP2A to enhance the phosphatase activity (Zhang *et al.*, 2007). Increased PP2A dephosphorylates TH-Ser40 and thereby reduces TH activity. In the present study, we observed that acute Mn exposure increases TH activity and TH-Ser40 phosphorylation. However, pretreatment with the PKC δ -specific inhibitor rottlerin did not block Mn-induced increases in TH activity, suggesting that PKC δ does not play a role in an acute-Mn treatment paradigm. A recent study showed increased TH activity without any increase in TH protein upregulation (Baek *et al.*, 2007). During chronic Mn treatment, we noted decreased TH activity with no significant change in TH-Ser40 phosphorylation. Interestingly, the PKC δ inhibitor rottlerin restored the TH activity in Mn-treated cells to that of untreated control cells, suggesting that PKC δ may regulate TH activity. It is also possible that Mn may mediate its effect by regulation the phosphorylation status of TH-associated proteins such as α -synuclein, 14-3-3 and PP2A because PKC δ has been shown to interact with these proteins (Zhang *et al.*, 2007; Wang *et al.*, 2009). Recently, Xing *et al.* showed Mn is also required for PP2A activity in which PP2A-specific methylsterase directly binds to the active site of PP2A leading to its inactivation by evicting the manganese ions that are required for the phosphatase activity of PP2A (Xing *et al.*, 2008).

Furthermore, chronic Mn treatment increased PKC δ activity and PP2A activity, consistent with our previous finding of synergistic interaction between PKC δ activity and PP2A. A recent study by Bevilaqua *et al.* demonstrated 1.5 mM Mn can mediate dephosphorylation of TH-Ser40 by activating PP2A in *in vitro* experiments (Bevilaqua *et al.*, 2003). Of note, the Mn concentration used in the Bevilaqua study was 1000-fold higher than the concentration used in this study. While our manuscript was under review, Posser *et al.* demonstrated that Mn induced a sustained Ser40 phosphorylation of TH and increased TH activity in pheochromocytoma cells (PC12 cells) (Posser *et al.*, 2009). The sustained Ser40 phosphorylation may be due to the 10–100-fold higher concentrations of Mn used in PC12 cell model. Together with the observation that PKC δ inhibition can restore both TH activity and PP2A activity to levels present in control cells, our study suggests that Mn-induced reduction in TH activity may in part be mediated by the PKC δ -PP2A signaling pathway.

The functional consequences of biphasic alterations of TH activity during Mn exposure, an increased TH activity during acute Mn-treatment and decreased TH activity during chronic Mn treatment, observed in this study need to be examined in future studies in animal models. Previous studies in animal models have shown some significant changes in the nigrostriatal dopaminergic system. A recent study by Liu et al. showed that chronic administration of large doses of Mn (100 mg/kg) for 8 weeks via oral gavage in mice depletes striatal dopamine level by 50% without inducing injury to dopaminergic neurons in nigra (Liu *et al.*, 2006). Guilarte et al. showed that Mn-exposed non-human primates exhibited subtle motor function deficits while apparently retaining an intact but dysfunctional nigrostriatal DA system (Guilarte *et al.*, 2006). Very recently, Guilarte et al. reported that chronic Mn exposure in primates significantly reduces amphetamine-induced dopamine release, as measured by the PET imaging procedure (Guilarte *et al.*, 2008). The authors also found reduced dopamine and 3,4-dihydroxyphenylacetic acid in the caudate putamen of Mn-treated monkeys without any significant change in the nigral dopaminergic neurons. Inhibition of TH activity observed in the present study might explain the reduced tissue dopamine levels and decreased stimulated-dopamine release observed in animal models of Mn toxicity. The differential alterations in TH activity observed in our study also may be modeling the toxicity occurring in human manganism cases resulting in neurological symptoms that are distinct in three stages (initial, intermediate and late).

In conclusion, we provide novel evidence that acute and chronic Mn exposures induce differential alterations in TH enzyme activity. Acute Mn exposure increased TH activity, whereas chronic Mn treatment decreased TH activity. Chronic Mn treatment also induced activation of PKC δ , which in turn activated PP2A, resulting in decreased TH activity due to dephosphorylation of TH-Ser40. Our results suggest that Mn-induced decreases in TH activity mediated through PKC δ and PP2A may explain the dopaminergic deficits observed during chronic Mn exposure.

Acknowledgments

This work is supported in NIH grants ES10586 and NS 386344. The support from W. Eugene and Linda Lloyd Endowment to AGK is also acknowledged. We would like to acknowledge Keri Henderson and Mary Ann deVries for their assistance in the preparation of this manuscript.

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Fig. 1.

Effect of acute Mn treatment on TH activity in differentiated N27 cells. Differentiated N27 cells were incubated with 3 or 10 μ M MnCl₂ for 3 h with or without 3 μ M rottlerin. For measurement of TH activity, cells were exposed to 2 mM NSD-1015 for 1 h prior to MnCl₂ treatment. Cells were lysed after treatment and extracts were used for determining L-DOPA levels by HPLC. **A**, Data are presented as pg DOPA/ μ g protein; **B**, data are presented as percentage of control. The data represent a mean + SEM of six to eight individual measurements. Asterisks (* p <0.05) indicate significant differences between Mn treated cells and control cells.

Fig. 2.

Effect of acute Mn treatment on TH phosphorylation level in differentiated N27 cells. Differentiated N27 cells were exposed to 3 or 10 μ M MnCl₂ for 3 h. Cell extracts were prepared and separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. TH antibody (mouse, 1:1000) and phospho-specific antibodies directed against P-TH-Ser40 (rabbit, 1:1000) were used for immunoblotting. To confirm equal protein loading in each lane, the membranes were reprobbed with β -actin antibody. The immunoblots were visualized using Amersham's ECL detection agents. Densitometric analysis of 60 kDa P-TH-Ser40 bands represents the mean $\pm$ SEM from three separate experiments (* p < 0.05, ** p < 0.01).

Fig. 3.

Cytotoxicity of acute MnCl₂ treatment in differentiated N27 cells. Differentiated N27 cells were treated with 3 µM or 10 µM MnCl₂ for 3 h. The effect of acute manganese treatment on cell death was quantified by Sytox Green fluorescence assay. The intensity of fluorescence was measured by fluorescence plate reader and shown as % of control. N27 cells exposed to 100 µM H₂O₂ for 3 h were used as a positive control. The data represent a mean + SEM of four individual measurements. Asterisks (***) indicate significant differences between H₂O₂-treated cells and control cells.

Fig. 4.

Effect of chronic MnCl₂ on TH activity and phosphorylation levels in differentiated N27 cells. Differentiated N27 cells were incubated with 0.1 μM, 0.3 μM or 1 μM MnCl₂ for 24 h. For measurement of TH activity, cells were exposed to 2 mM NSD-1015 for 1 h prior to MnCl₂ treatment. Cells were lysed after treatment, and extracts were used to determine L-DOPA levels by HPLC. **A**, TH activity expressed as pg DOPA/μg protein; **B**, TH activity expressed as percentage of control. The data represent a mean + SEM of six to eight individual measurements. Asterisks (*p<0.05) indicate significant differences between MnCl₂-treated cells and control cells. **C**, Western blot of P-TH-Ser40. Cell extracts from control and 1 μM MnCl₂-treated N27 cells were prepared and separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. TH antibody and phospho-specific antibodies directed against P-TH-Ser40 were used for immunoblotting. To confirm equal protein loading in each lane, the membranes were reprobed with β-actin antibody. The immunoblots were visualized using Amersham's ECL detection agents.

Fig. 5.

Cytotoxicity of chronic MnCl₂ treatment in differentiated N27 cells. Differentiated N27 cells were treated with 0.1 μM, 0.3 μM or 1 μM MnCl₂ for 24 h. The effect of chronic manganese treatment on cell death was quantified by Sytox Green fluorescence assay. The intensity of fluorescence was measured by fluorescence plate reader and shown as % of control. N27 cells exposed to 100 μM H₂O₂ for 3 h were used as a positive control. The data represent a mean + SEM of four individual measurements. Asterisks (***) indicate significant differences between H₂O₂-treated cells and control cells.

Fig. 6. Effects of PKC δ inhibition on MnCl₂-induced reduction of TH activity in differentiated N27 cells. Differentiated N27 cells were treated with 1 μ M MnCl₂ or cotreated with 3 μ M rottlerin for 24 h. **A**, TH activity: Cells were pretreated with 2 mM NSD-1015 for 1 h before MnCl₂ treatment and lysed. Extracts were used for determining L-DOPA levels by HPLC. The data represent a mean + SEM of six to eight individual measurements. Asterisks (** p <0.01) indicate significant differences between MnCl₂-treated cells and control cells. **B**, PKC δ activity: cell extracts from the above treatment were used in the immunoprecipitation kinase assay. Recombinant PKC δ protein was used as a positive control. The bands were quantified by a PhosphoImager after scanning the dried gel and are expressed as a percentage of control. The data represent a mean + SEM of three individual measurements. Asterisks (** p <0.01) indicate significant differences between MnCl₂ treated and control cells.

Fig. 7.

Effect of Mn on PP2A activity in dopaminergic cells. Differentiated N27 cells were treated with 1 μ M MnCl₂ or cotreated with 3 μ M rottlerin for 24 h. Then cells were lysed and PP2A enzyme activity was measured using a serine/threonine phosphatase assay kit from Promega. The data represent a mean + SEM of four to six individual measurements. Asterisks (* p <0.05) indicate significant differences between MnCl₂ treated and control cells.