Manganese Induces Tau Hyperphosphorylation through the Activation of ERK MAPK Pathway in PC12 Cells

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Manganese has long been known to induce neurological degenerative disorders. Emerging evidence indicates that hyperphosphorylated tau is associated with neurodegenerative diseases, but whether such hyperphosphorylation plays a role in manganese-induced neurotoxicity remains unclear. To fill this gap, we investigated the effects of manganese on tau phosphorylation in PC12 cells. In our present research, treatment of cells with manganese increased the phosphorylation of tau at Ser199, Ser202, Ser396, and Ser404 as detected by Western blot. Moreover, this manganese-induced tau phosphorylation paralleled the activation of extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK). The mitogen-activated protein kinase kinase-1 (MEK1) inhibitor PD98059, which inhibits the activation of ERK MAPK, partially attenuated manganese-induced tau hyperphosphorylation and cytotoxicity. Moreover, the activation of ERK MAPK was involved in the activation of glycogen synthase kinase-3β (GSK-3β) kinase, which also contributed to the hyperphosphorylation of tau and the cytotoxicity in PC12 cells induced by manganese. Taken together, we found for the first time that the exposure to manganese can cause the hyperphosphorylation of tau, which may be connected with the activation of ERK MAPK.

Key Words: manganese; tau; PC12 cell; ERK MAPK; GSK-3β.

Manganese is an essential trace element required for a series of physiological functions, and it is important for brain development (Takeda et al., 1999). But too much absorption of manganese can cause neurotoxicity in human beings (Cai et al., 2007; Liu et al., 2009). Chronic occupational exposure to high levels of manganese can cause its accumulation in basal ganglia and result in manganism, a condition characterized by tremors, bradykinesia, rigidity, dystonia, specific gait disturbances, and psychosis (Burton and Guilarte, 2009; Mergler and Baldwin, 1997). Increased manganese concentrations in the brain can occur under a variety of conditions, such as occupational, iatrogenic, medical, and environmental exposures (Burton and Guilarte, 2009). First described by Couper in 1837 (Couper, 1837), manganism can be found in welding workers with chronic manganese exposure (McMillan, 2005). Millions of workers worldwide are exposed to toxic levels of manganese (Checkoway, 2010). The introduction of methylcyclopentadienyl manganese tricarbonyl as an additive to unleaded gasoline in some countries may increase human exposure to manganese (Landrigan et al., 2005). Though there are some differences between manganism and Parkinson’s disease (PD) (Calne et al., 1994; Salehi et al., 2006), some researchers believe that manganese exposure is a PD environmental risk factor (Mergler and Baldwin, 1997). Recently, the accumulation of manganese has been found to be an etiology of PD in the general population of China (Fukushima et al., 2010). Up to present, the molecular mechanisms by which manganese induces neurotoxicity are not yet well understood.

Tau, a major member of microtubule-associated proteins (MAPs), is predominantly expressed in neurons. It is detected abundantly in the axons of mature neurons (Hirokawa et al., 1996). The phosphorylation of tau is a modification that can affect a total of 79 residues in the longest tau isoform in the central nervous system of 441 residues (Avila, 2006). The phosphorylation of tau and its formation of neurofibrillary tangles (NFTs), which can be regarded as an important characteristic of Alzheimer’s disease (AD) (Alvarez et al., 2002). NFTs are intraneuronal aggregates of fibrils with the appearance of straight and paired helical filaments, which are mainly constituted by hyperphosphorylated tau protein. Indeed, tau hyperphosphorylation is one of the earliest events detected in the brains of patients with AD (Alvarez et al., 2002). The hyperphosphorylation of tau has also been found to be involved...
in PD pathogenesis (Muntane et al., 2008). In recent years, hyperphosphorylated tau protein has been found to be involved in the neurotoxicity of several neurotoxins, such as β-amyloid (Olivieri et al., 2001), mercury (Olivieri et al., 2000), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Wang et al., 2007). Moreover, several kinases, including glycogen synthase kinase-3 (GSK-3) and mitogen-activated protein kinases (MAPKs), are responsible for the phosphorylation of tau and the formation of neurofilaments (Lee et al., 2005).

Tau hyperphosphorylation has been shown to be involved in neurodegenerative pathogenesis. However, whether it plays a role in manganese-induced neurotoxicity is still unknown. In the present study, we investigated whether manganese could induce the hyperphosphorylation of tau in PC12 cells. Moreover, the relation between MAPKs and the change of tau phosphorylation in response to manganese was also evaluated. The results may further our insights into the mechanisms of manganese-induced neurotoxicity.

MATERIALS AND METHODS

Cell culture. Differentiated PC12 cells were obtained from Cell Bank of Chinese Academy of Sciences and maintained in High-glucose Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% heat-inactivated newborn calf serum (Sijiqing Biotech, China), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a water-saturated atmosphere of 5% CO2 at 37°C. Medium was replenished every 48 h. Manganese chloride (MnCl2), mitogen-activated protein kinase kinase-1 (MEK1) inhibitor PD98059, lithium chloride (LiCl), and other reagents in the molecular studies were obtained from Sigma (St Louis, MO).

Cell viability assay. Cell viability was measured by using methyl thiazolyl tetrazolium (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). PC12 cells (5 × 104 cells per well) were seeded into 96-well microtiter plates. Following treatment, 20 μl MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for 4 h at 37°C. Then, the supernatant layer was removed, and 150 μl of dimethyl sulfoxide was added into each well. The plates were shaken vigorously for 10 min to ensure complete solubilization. MTT metabolism was quantitated spectrophotometrically at 490 nm in a microplate reader.

Flow cytometric evaluation of apoptosis. Flow cytometry was used to assess the membrane and nuclear events during apoptosis. The assay was performed with a two-color analysis of fluorescein isothiocyanate (FITC)-labeled Annexin V binding and propidium iodide (PI) uptake. Positioning of quadrants on Annexin V/PI dot plots was performed and live cells (Annexin V−/PI−), early/primary apoptotic cells (Annexin V+/PI−), late/secondary apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V−/PI+) were distinguished. Therefore, the total apoptotic proportion included the percentage of cells with fluorescence Annexin V+/PI− and Annexin V+/PI+ (Wu et al., 2007). After treatment, PC12 cells were harvested, washed, and double stained by using an Annexin V-FITC apoptosis detection kit. Samples were incubated at room temperature for 15 min in the dark with Annexin V and PI and quantitatively analyzed by a FACScalibur flow cytometer (Beckon Dickinson).

Western blot analysis. After terminating the experiments, protein from different groups was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Following transfer, non-specific binding sites were blocked by immersing the membranes in 5% bovine serum albumin in PBS at room temperature, and then incubated with the primary antibodies. Subsequently, the membranes were incubated with appropriate secondary antibodies (Zhongshan Biotechnology, China). All secondary antibodies were horseradish peroxidase conjugated. Enhanced chemiluminescence (ECL) Western Blotting Substrate (Pierce) was used to detect the immunoreactive signals with an ECL-based FluorChem FC2 image system (Alpha Innotech). Primary antibodies used were phospho-Erk1/2 (Thr202/Tyr204) antibody (Cell Signaling), total-Erk1/2 antibody (Cell Signaling), phospho-p38 (Thr180/Tyr182) antibody (Cell Signaling), total-p38 antibody (Cell Signaling), phospho-stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (Thr183/Tyr185) antibody (Cell Signaling), total-SAPK/JNK antibody (Cell Signaling), tau-1 antibody (Invitrogen), tau-pSer396 antibody (Invitrogen), tau-pSer404 antibody (Invitrogen), tau-5 antibody (Invitrogen), phospho-GSK-3β (Ser9) antibody (Cell Signaling), and total-GSK-3β antibody (Cell Signaling). All Western blot analyses were performed in triplicate. Then FluorChem FC2 software was used to analyze the gray value of the protein expression in each group.

Statistical analysis. The data are presented as means ± SDs. Statistical analysis for comparison of mean values was performed by ANOVA followed by Dunnett’s post hoc test using SPSS 11.0 for windows. Probability values lower than 0.05 were considered to be statistically significant.

RESULTS

Manganese-Induced Cytotoxicity in PC12 Cells

As an essential trace element, manganese is required for many ubiquitous enzymatic reactions (Dobson et al., 2004). However, manganese can accumulate in certain brain regions following elevated exposures, and manganese-induced neurotoxicity can ensue (Dobson et al., 2004). In our present study, we first confirmed manganese-induced toxicity. PC12 cells were treated with MnCl2 of different concentrations (0, 100, 300, and 500μM) for 24 h. Then, the cell viability of PC12 cells was assessed by MTT assay. After treatment of 24 h, 100, 300, and 500μM MnCl2 reduced cell viability to 90.3 ± 5.3%, 68.7 ± 7.1%, and 59.9 ± 6.4% of the control level (Fig. 1A). Thus, manganese treatment reduced the cell survival. We further determined the apoptosis of PC12 cells by flow cytometric assay. As shown in Figure 1B, manganese treatment also induced the apoptosis of PC12 cells. After the treatment by MnCl2 of 0, 100, 300, and 500μM for 24 h, the percentages of apoptotic cells were 4.2 ± 2.3%, 14.9 ± 3.2%, 36.1 ± 6.7%, and 36.9 ± 4.3%, respectively (Fig. 1B). Taken together, the treatment of PC12 cells with MnCl2 induced cytotoxicity in a dose-dependent manner. Thus, we confirmed manganese-induced cytotoxicity in PC12 cells. The concentration of 300μM was chosen to continue the following researches.

Manganese Induced the Hyperphosphorylation of tau in PC12 Cells

The hyperphosphorylation of tau has been found to be involved in several neurodegenerative diseases, such as AD (Alvarez et al., 2002) and PD (Muntane et al., 2008). However, it is still unclear whether manganese could induce the hyperphosphorylation of tau. In the present study, the phosphorylation levels of tau protein at four sites in PC12 cells treated by manganese were determined by using Western blot. Tau-1
antibody recognizes unphosphorylated level of Ser199 and Ser202 sites, whereas tau-pSer396 and tau-pSer404 antibodies recognize phosphorylated tau protein at other two sites. Tau-5 antibody recognizes the total level of tau at the middle part of the molecule. When PC12 cells were treated with 300µM MnCl2 for different time periods, the phosphorylation level increased with the prolongation of exposure time (Fig. 2A). The addition of MnCl2 to PC12 cells also induced a dose-dependent increase of tau phosphorylation level at the four sites. After incubation with various concentrations of MnCl2 (100, 300, and 500µM) for 24 h, MnCl2 induced the phosphorylation of tau at the four sites in a concentration-dependent manner (Fig. 2B). These results suggest that manganese can induce tau phosphorylation in both time- and dose-dependent manners.

Manganese-Induced ERK MAPK Phosphorylation in PC12 Cells

Because MAPK cascades play an important role in the regulation of tau phosphorylation, we examined the levels of p-ERK, p-p38, and p-JNK in manganese-treated PC12 cells. Our results demonstrated that the p-ERK1/2 levels in PC12 cells were significantly induced after manganese treatment. After incubation with various concentrations of MnCl2 (100, 300, and 500µM) for 0.5, 1.5, and 3 h, MnCl2 induced the phosphorylation of p-ERK1/2 in a concentration-dependent manner (Fig. 3). However, there was no obvious induction of p-p38 and p-JNK. So we can draw a conclusion that manganese can induce the activation of ERK MAPK while inducing the hyperphosphorylation of tau. Especially at 3 h time point, both the activation of ERK MAPK and the hyperphosphorylation of tau were induced dramatically.

The Inhibition of ERK MAPK Attenuated the Hyperphosphorylation of tau and the Cytotoxicity in PC12 Cells Induced by Manganese

ERK MAPK has been shown to be involved in the hyperphosphorylation of tau protein, but whether it plays a role in manganese-induced tau hyperphosphorylation is still unclear. So we used MEK1 inhibitor PD98059 to inhibit the activation of ERK MAPK kinase and to investigate the possible role of this pathway in manganese-induced tau hyperphosphorylation. In the present study, the pretreatment of 50µM PD98059 for half hour dramatically inhibited the hyperphosphorylation of tau protein at Ser396 site. However, there were only marginal effects on the hyperphosphorylation of tau at Ser199, Ser202, and Ser404 sites (Fig. 4A). So, in our present conditions, manganese induced the hyperphosphorylation of tau, especially at Ser396 site, in PC12 cells through the activation of ERK MAPK. Besides the inhibition of tau hyperphosphorylation, PD98059 also inhibited manganese-induced cytotoxicity. The cell viability of PC12 cells was determined by using MTT assay. After incubation with 300µM MnCl2 for 24 h, manganese reduced the cell viability to 62.3 ± 7.2% of the control group. PD98059 markedly protected PC12 cells against the reduction of cell viability induced by
manganese. Pretreatment of cells with 50 μM PD98059 for half an hour raised the cell viability to 89.3 ± 10.7% of the control group (Fig. 4B). PD98059 also inhibited manganese-induced apoptosis. After incubation with 300 μM MnCl₂ for 24 h, the percentage of apoptotic cells was 31.2 ± 4.1%. PD98059 decreased the percentage of apoptotic cells from 31.2 ± 4.1 to 22.5 ± 3.7%, as shown by flow cytometric results (Fig. 4C). Taken together, these results show that the activation of ERK1/2 MAPK may play an important role in manganese-induced tau hyperphosphorylation and cytotoxicity.

**FIG. 2.** Manganese-induced hyperphosphorylation of tau in PC12 cells. The phosphorylation levels of tau protein at four sites in PC12 cells were determined by using Western blot. Tau-1 antibody recognizes unphosphorylated level of Ser199 and Ser202 sites, tau-pSer396 and tau-pSer404 antibodies recognize phosphorylated tau protein at other two sites, and tau-5 antibody recognizes the total level of tau protein. (A) PC12 cells were treated with 300 μM MnCl₂ for 0, 0.5, 1, 3, and 6 h. (B) PC12 cells were treated with 0, 100, 300, and 500 μM MnCl₂ for 6 h. *p < 0.05 compared with the control group; **p < 0.01 compared with the control group.

**FIG. 3.** Manganese-induced ERK MAPK activation in PC12 cells. The phosphorylation states of MAPKs in PC12 cells were determined by using Western blot. PC12 cells were treated by 0, 100, and 300 μM MnCl₂ for 0.5, 1.5, and 3 h (30, 90, and 180 min). After treatment, the cells were washed with PBS three times and harvested as described in experimental procedures. The activation of MAPKs was determined by using anti-phospho-MAPK antibodies. The total MAPKs were similarly determined using anti-phosphorylation–independent MAPK antibodies. *p < 0.05 compared with the control group; **p < 0.01 compared with the control group.
The Activation of ERK MAPK Was Involved in the Activation of GSK-3β Kinase, Which Also Contributed to the Hyperphosphorylation of tau and the Cytotoxicity in PC12 Cells Induced by Manganese

GSK-3β is an important kinase involved in the phosphorylation of tau (Mulot et al., 1994; Tatebayashi et al., 2004). LiCl, an inhibitor of GSK-3β, has long been confirmed to inhibit tau phosphorylation (Hong et al., 1997). We further examined the activity of GSK-3β after manganese treatment. Because the activity of GSK-3β is inhibited by the phosphorylation at its Ser9 site (Cross et al., 1995), an antibody against Ser9 was used. Our results demonstrated that the p-Ser9-GSK-3β level in PC12 cells was reduced in a time-dependent manner after the treatment by 300 μM MnCl₂. Obvious GSK-3β dephosphorylation can be observed after 3 h of manganese treatment (Fig. 5A). So, manganese enhanced the activity of GSK-3β. Because the obvious activation of ERK MAPK by 300 μM MnCl₂ happened as early as 30 min after treatment (Fig. 3), which was earlier than the activation of GSK-3β, we further determined whether there was any connection between the activation of ERK MAPK and the later activation of GSK-3β. In the present study, manganese-induced activation of GSK-3β at 6 h time point was dramatically inhibited by the pretreatment of 50 μM PD98059 (PD) or vehicle for 0.5 h before the treatment of MnCl₂ (300 μM) for 24 h. Data are expressed as percentage of cell survival compared with the control group and mean ± SD of experiments. **p < 0.01 compared with the control group; #p < 0.01 compared with 300 μM MnCl₂ group. (B) The effect of PD98059 on manganese-induced cell viability inhibition, as assessed by MTT method. PC12 cells were pretreated with 50 μM PD98059 (PD) or vehicle for 0.5 h before the treatment of MnCl₂ (300 μM) for 24 h and then the cell apoptosis was determined by a flow cytometric method as described previously. The total apoptotic proportion included the percentage of cells with fluorescence Annexin V+/PI− and Annexin V+/PI+. **p < 0.01 compared with the control group; #p < 0.05 compared with 300 μM MnCl₂ group.

FIG. 4. The inhibition of ERK MAPK attenuated the hyperphosphorylation of tau and the cytotoxicity induced by manganese. (A) The effect of PD98059 on manganese-induced tau hyperphosphorylation. PC12 cells were pretreated for 0.5 h with or without PD98059 (PD) before the treatment of MnCl₂ for additional 6 h. After treatment, the phosphorylation levels of tau protein at four sites in PC12 cells were determined by using Western blot as described previously. *p < 0.05 compared with the control group; **p < 0.01 compared with the control group; #p < 0.01 compared with 300 μM MnCl₂ group. (B) The effect of PD98059 on manganese-induced cell viability inhibition, as assessed by MTT method. PC12 cells were pretreated with 50 μM PD98059 (PD) or vehicle for 0.5 h before the treatment of MnCl₂ (300 μM) for 24 h. Data are expressed as percentage of cell survival compared with the control group and mean ± SD of experiments. **p < 0.01 compared with the control group; #p < 0.01 compared with 300 μM MnCl₂ group. (C) The effect of PD98059 on manganese-induced cell apoptosis. PC12 cells were treated with 50 μM PD98059 (PD) or vehicle for 0.5 h before exposure to MnCl₂ (300 μM) for 24 h and then the cell apoptosis was determined by a flow cytometric method as described previously. The total apoptotic proportion included the percentage of cells with fluorescence Annexin V+/PI− and Annexin V+/PI+. **p < 0.01 compared with the control group; #p < 0.05 compared with 300 μM MnCl₂ group.
Furthermore, LiCl also inhibited manganese-induced cytotoxicity. In MTT assay, the incubation with 300 μM MnCl₂ for 24 h reduced the cell viability to 58.9 ± 3.2% of the control group. LiCl raised the cell viability from 58.9 ± 3.2 to 90.3 ± 7.9% of the control group (Fig. 6B). LiCl also inhibited manganese-induced apoptosis. After incubation with 300 μM MnCl₂ for 24 h, the percentage of apoptotic cells was 28.3 ± 5.4%. LiCl decreased the percentage of apoptotic cells caused by manganese from 28.3 ± 5.4% to 6.2 ± 2.9% (Fig. 6C). So, in our present conditions, manganese induced the activation of GSK-3β in PC12 cells through the activation of ERK MAPK, which was also involved in tau hyperphosphorylation and cytotoxicity induced by manganese.

**DISCUSSION**

In the present investigation, we found for the first time that the exposure to manganese can cause the hyperphosphorylation of tau. Our data also show that the activation of ERK MAPK pathway may be involved in such hyperphosphorylation induced by manganese.

Tau protein is the primary component of the intracellular filamentous deposits found in AD brain and also in a family of neurodegenerative diseases called “tauopathies” (Chun and Johnson, 2007). As a group of neuronal MAPs, tau plays a key role in regulating microtubule dynamics, axonal transport, and neurite outgrowth. All these functions of tau are modulated by site-specific phosphorylation (Johnson and Stoothoff, 2004). In pathological conditions such as AD and PD, tau is abnormally phosphorylated, which contributes to its dysfunction (Mi and Johnson, 2006). Increasing evidences show that the disruption in the normal phosphorylation state of tau followed by conformational changes plays a key role in the pathogenic events that occur in several neurodegenerative diseases (Mi and Johnson, 2006). It has also been reported that several neurotoxins can induce their neurotoxicities through tau hyperphosphorylation. MPTP is an important environmental dopaminergic neurotoxin (Dauer et al., 2002). It has been reported that both the treatment of primary mesencephalic neurons with 1-methyl-4-phenylpyridinium, the active metabolite of MPTP, and subchronic treatment of mice with MPTP result in selective dose-dependent hyperphosphorylation of tau (Duka et al., 2006). However, it is still unclear whether manganese could induce tau hyperphosphorylation. In the present study, the induction of tau hyperphosphorylation in PC12 cells was caused by manganese treatment. Our data show that manganese could induce the hyperphosphorylation of tau at Ser199, Ser202, Ser396, and Ser404 in both time- and dose-dependent manners.

Several kinases have been found to be involved in the hyperphosphorylation of tau protein, such as GSK-3, cyclin-dependent kinase-5, MAPKs, casein kinase, calcium calmodulin-dependent kinase II, microtubule affinity-regulating kinase, protein kinase A (cyclic adenosine monophosphate-dependent protein kinase), and others (Churcher, 2006). ERK is an important member of MAPK family (Zheng et al., 2008). It is now well established that neuronal ERK MAPK cascades play important roles in synaptic plasticity, memory formation, and some other important neurological functions (Impay et al., 1999). The activation of ERK MAPK has also been found to play a critical role in some neurodegenerative diseases such as AD and PD (Kim and Choi, 2010). Previous studies show that ERK MAPK is connected with manganese-induced neurotoxicity (Hirata, 2002; Ito et al., 2006), but the mechanism is still...
not clear. ERK has also been suggested to be important in modulating tau phosphorylation (Dehvari et al., 2008; Pei et al., 2002). In the present research, we confirmed manganese-induced activation of ERK MAPK. Furthermore, the inhibition of this kinase attenuated the hyperphosphorylation of tau protein. So, manganese may induce the hyperphosphorylation of tau through the activation of ERK MAPK. However, the present data show that PD98059 dramatically inhibited the hyperphosphorylation of tau protein at Ser396 site, and there were only marginal effects on the hyperphosphorylation of tau at Ser199, Ser202, and Ser404 sites. This is consistent with that the cytotoxicity induced by manganese was only partly alleviated by the inhibition of ERK MAPK.

GSK-3 was initially identified as an enzyme that regulated glycogen synthesis in response to insulin (Welsh et al., 1996). It is expressed abundantly in neurons and is involved in tau phosphorylation (Tatebayashi et al., 2004). There are two mammalian GSK-3, GSK-3α and GSK-3β. They are structurally similar but functionally nonidentical. The activation of GSK-3β has been reported to be involved in several neurotoxin-induced neurotoxicities, such as amyloid-β (Hoppe et al., 2010), ethanol (Luo, 2009), MPTP (Petit-Paitel et al., 2009), 2,3,7,8-tetrachlorodibenzo-p-dioxin (Sul et al., 2009), and mercury (Olivieri et al., 2000). More importantly, the application of GSK-3β inhibitors has been proven to be a promising strategy against both neurodegenerative diseases and neurotoxin-induced neurotoxicities, which is connected with the inhibitory effects on tau hyperphosphorylation (Koh et al., 2008; Mazanetz and Fischer, 2007; Sul et al., 2009). In our present study, manganese induced the activation of GSK-3β. Such activation was also connected with the activation of ERK MAPK, as the inhibition of ERK MAPK alleviated the activation of GSK-3β in response to manganese. Moreover, an inhibitor of GSK-3β, LiCl, can inhibit both the hyperphosphorylation of tau and the cytotoxicity induced by manganese. These results further confirmed the role of ERK MAPK in manganese-induced tau hyperphosphorylation. The regulatory role of ERK MAPK on GSK-3β was first spotted in

FIG. 6. The inhibition of GSK-3β also attenuated the hyperphosphorylation of tau and the cytotoxicity induced by manganese. (A) The effect of LiCl on manganese-induced tau hyperphosphorylation. PC12 cells were pretreated for 0.5 h with or without 50mM LiCl before the treatment of MnCl₂ for additional 6 h. After treatment, the phosphorylation of tau protein at four sites in PC12 cells was determined by using Western blot as described previously. *p < 0.05 compared with the control group; **p < 0.01 compared with the control group; ###p < 0.01 compared with 300 μM MnCl₂ group. (B) The effect of LiCl on manganese-induced cell viability inhibition, as assessed by MTT method. PC12 cells were pretreated with 50mM LiCl or vehicle for 0.5 h before the treatment of MnCl₂ (300μM) for 24 h. *p < 0.01 compared with the control group; ###p < 0.01 compared with 300μM MnCl₂ group. (C) The effect of LiCl on manganese-induced cell apoptosis. PC12 cells were treated with 50mM LiCl or vehicle for 0.5 h before exposure to MnCl₂ (300μM) for 24 h and then the cell apoptosis was determined by a flow cytometric method as described previously. The total apoptotic proportion included the percentage of cells with fluorescence Annexin V+/PI– and Annexin V+/PI+. **p < 0.01 compared with the control group; ###p < 0.01 compared with 300 μM MnCl₂ group.
1998 (He et al., 1998) and was further confirmed by several other researches (Dozza et al., 2004; Kumar et al., 2010; Luo, 2009; Rasola et al., 2010). However, in most cases, ERK MAPK is responsible for the phosphorylation and the further inhibition of GSK-3β. As a serine/threonine protein kinase, it is interesting that the activation of ERK MAPK led to the dephosphorylation of GSK-3β at Ser9 in our present study. There may be some mediators by which ERK MAPK led to GSK-3β activation.

In our present research, LiCl only partly inhibited manganese-induced hyperphosphorylation of tau. It dramatically inhibited the hyperphosphorylation of tau protein at Ser396 site while it had only marginal effects on tau phosphorylation at Ser199, Ser202, and Ser404 sites, which is similar to the effects of PD98059. The effects on kinases and tau phosphorylation may be neurotoxin dependent. As we have mentioned above, a series of kinases are involved in the phosphorylation of tau. Also, GSK-3β has been found to be connected with the hyperphosphorylation of tau at more than 10 sites (Liu et al., 2002, 2003; Sengupta et al., 2006). So, there may be other kinases responsible for manganese-induced hyperphosphorylation of tau. Furthermore, more sites than those we have investigated may be involved in manganese-induced cytotoxicity. Taken together, the activation of ERK MAPK was involved in the activation of GSK-3β kinase, which contributed to manganese-induced tau hyperphosphorylation and cytotoxicity in PC12 cells.

To summary, our present study shows that manganese can induce the hyperphosphorylation of tau, which is dependent on the activation of ERK MAPK pathway. These observations may further our understanding of manganese-induced neurotoxicity. However, more work will be done to elucidate the mechanisms implied in the manganese-induced tau hyperphosphorylation. Because of the limit of in vitro model (e.g., the concentrations used in in vitro studies are always higher than both the blood manganese concentrations in exposed workers, Meyer-Baron et al., 2009, and the concentrations in animal models, Reaney et al., 2006; Zhao et al., 2009), further in vivo studies may be helpful in the future researches.

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MANGANESE-INDUCED TAU HYPERPHOSPHORYLATION


