Differential Role of Mitogen-Activated Protein Kinases in Response to Manganese Treatment in Substantia Nigra Dopaminergic Neurons

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Recent studies have provided evidence that exposure to manganese (Mn) induces Parkinson's disease (PD)-like symptoms. We investigated the mechanism of Mn neurotoxicity in the SN4741 dopaminergic (DA) neuronal cell line. The results indicated that the p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases were activated during DA cell death by $MnCl_2$. Moreover, p38 inhibition with SB203580, a specific inhibitor of p38, induced more toxic effects in $MnCl_2$-treated cells. Among the p38 subfamily members, p38α attenuated the caspase-3 activation and cell death induced by $MnCl_2$. However, the expression of JNK stimulated the activity of caspase-3 and mediated the Mn-induced cell death. These results suggest that there are multiple pathways in $MnCl_2$-induced DA neuronal cell death.

Key words —— mitogen-activated protein kinases, p38α, p38β, manganese chloride, dopaminergic cells

INTRODUCTION

Manganese (Mn) intoxication, sometimes referred to as manganism, has long been proposed to be a risk factor for irreversible neurologic dysfunction.[1)] Mn can cross the blood-brain barrier (BBB) via a nonspecific divalent metal transporter-1 (DMT1) or transferrin receptor-mediated mechanism.[2)] In the human brain, Mn is accumulated in the globus pallidus, striatum, thalamus, and substantia nigra at higher levels than in other areas. All those areas are vulnerable to Mn toxicity. Mn-induced neuronal loss and depigmentation were significant in a post-mortem study.[3)] Mn plays an important role as a cofactor in many enzymatic reactions but it can cause parkinsonism at high concentrations.[1,4)]

Mn was labeled as a brain dysfunction inducing-environmental toxin by the World Health Organization because Mn is related to frequent industrial accidents. Many workers are exposed to airborne particles containing Mn in the several types of work place such as Mn mines, welding shops, and manufacturers of batteries, ferroalloys, paints, chlorine gas, or linoleum.[1,3,5)] Moreover, the antiknock gasoline additive methylcyclopentadienyl manganese tricarbonyl (MMT) elevates airborne levels of Mn and causes significant health threats to the general population.[5)] The homeostatic and biochemical mechanisms of Mn toxicity must be understood to design neurotoxicity prevention strategies.

Although Mn-induced parkinsonism has some distinguishing features from idiopathic Parkinson's disease (PD), there are also similarities, notably the presence of abnormal gait, expressionless face, bradykinesia, dysarthria, difficulty in walking, and lack of balance.[4)] The relationship between Mn neurotoxicity and PD is still debated, but at least Mn exposure is a risk factor for PD.[6,7)]

The mechanisms of Mn neurotoxicty remain unclear. Earlier reports suggested that Mn could enhance autooxidation of catecholamines and generate reactive oxygen species[8)] and inhibit mitochondrial aconitase activity and disrupt mitochondrial energy production in the brain.[9,10)] In addition, Mn has been found to increase N-methyl-D-aspartic acid (NMDA)-mediated excitotoxicity, alter calcium homeostasis, and lead to cell death.[11)] Mn may stimulate apoptosis or necrosis in PC12 cells as evidenced by increased DNA cleavage, activation of the c-Jun N-terminal kinase (JNK) pathway, and caspase-3 activation.[12,13)] Nevertheless, the exact mechanism of action of Mn neurotoxicity

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and the reason for the vulnerability of the substantia nigra is not known.

We previously established a dopaminergic (DA) neuronal cell line, SN4741, from transgenic mouse embryos with targeted immortalization of substantia nigra DA neurons.[14] Using this cell line, the present study was designed to determine the cell death pathways, especially the role of mitogen-activated protein (MAP) kinase pathways induced by Mn.

MATERIALS AND METHODS

Materials —— PD98059 and SB203580 were obtained from Calbiochem. MnCl, SP600125, and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). The Fugene 6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Antibodies against phospho-ERK1/2, phospho-JNK, and phospho-p38 were obtained from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). Polyclonal antibodies against the total forms of ERK1/2, JNK, and p38 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.).

Cell Culture and Treatments —— The DA neuronal SN4741 cells were cultured at 33°C as described previously[14] and subcultured every 2–3 days at approximately 70–80% confluency. To estimate cell viability, the 2,3-bis-(2-methoxy-4-nitro-t-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay was used in combination with the total cell counting, using trypan blue dye exclusion, as previously described.[15] Treatment chemicals dissolved in dimethyl sulfoxide (DMSO) were added at a maximum ratio of 1 : 1000 to the culture media. This concentration of DMSO was shown not to effect SN4741 cell viability.

Immunoblotting —— For immunoblot analysis, total cell proteins were prepared from the SN4741 cells grown under various experimental conditions. Protein lysate 20 μg was used for sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), transferred to poly(vinylidene fluoride) (PVDF) membranes, and then specific proteins were detected with the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, U.S.A.) as described.[14]

Transient Transfection and Caspase-3 Assay —— Flag-tagged cDNA encoding p38α (pcDNA3-p38α) and p38β (pcDNA3-p38β) were generously provided by Dr. Jiahuai Han.[16] Flag-tagged JNK1 cDNA was a kind gift from Dr. Roger J. Davis.[17] SN4741 cells were transiently transfected with either pcDNA3 (vector) or the various constructs described above, using the Fugene 6 transfection reagent. Plasmid pSVβ-gal encoding β-galactosidase was used to normalize variations in transfection efficiency. After 24 hr transfection, the cells were cultured for an additional 24 hr in low-serum (0.5%) medium containing $MnCl_2$ 500 μM. For small interfering (si) RNA treatment, siRNA duplex was delivered by the TransIT-TKO transfection reagent (Mirus, Madison, WI, U.S.A.). As previously described, siRNAs were synthesized with specific coding regions for the mouse p38α gene, 5′-UACCGAGAGUUGCGUCUGCdTdT-3′ and p38β gene, 5′-UACCGUGAGCUGCGCCUACdTdT-3′, respectively.[18] As a negative control, scrambled siRNA (Dharmacon Research, Lafayette, CO, U.S.A.) was also transfected into SN4741 cells. The effects of siRNA were evaluated 48–72 hr after transfection of siRNA duplex. Preparation of cell extracts and measurement of caspase-3 activity were performed as described previously.[19] Protein samples (10 μg) were incubated at 25°C with 200 μM of caspase-3 substrate (Ac-DEVD-pNA; Biomol, Plymouth Meeting, PA, U.S.A.). Formation of *p*-nitroaniline (*p*NA) from the reaction was measured at the wavelength of 405 nm. After recording data for 30 to 60 min, the activity was calculated as picomoles of substrate hydrolyzed per minute.

Statistical Analysis —— Data were analyzed using the GraphPad Prism data analysis program (GraphPad Software, San Diego, CA, U.S.A.). For comparison of statistical significance between two groups, the Student's *t*-test for paired and unpaired data was used. A p value of less than 0.05 was considered significant.

RESULTS

It was shown that Mn induces damage to various neuronal cells including DA neuronal cells,[9, 12, 13] but the details of the mechanisms have yet to be elucidated. Previous studies revealed that the MAP kinases are phosphorylated during neuronal cell death.[12, 20] Thus, in this study, SN4741 cells were treated with $MnCl_2$ 500 μM to examine whether $MnCl_2$ affects MAP kinase family member protein expression. Immunoblot analysis revealed that the

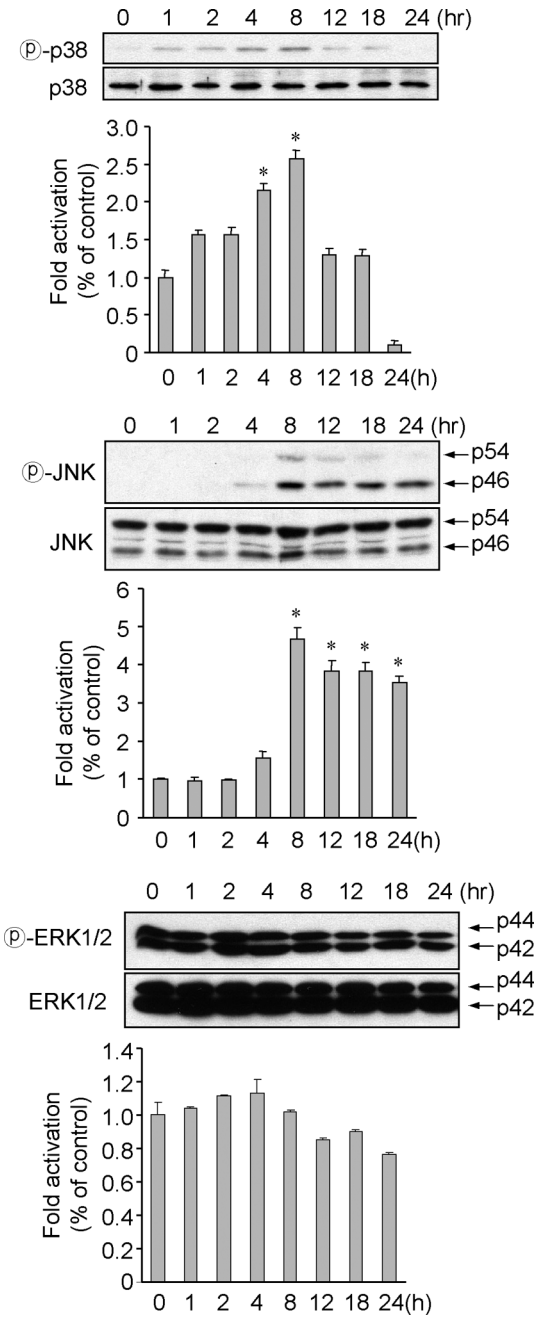

Fig. 1. Mn-Induced MAP Kinase Activation in SN4741 Cells

After $MnCl_2$ 500 μM treatment, SN4741 cell lysates were prepared at the indicated time points. Lysates (20 μg) were analyzed by immunoblotting with antibodies against p38, JNK, and ERK1/2 proteins and their active (phosphorylated) forms. The immunoblot shown is representative of four different experiments. Relative densitometric density was assessed as the ratio against the value of control (0 hr). All values are mean ± SEM. $^*p < 0.05$.

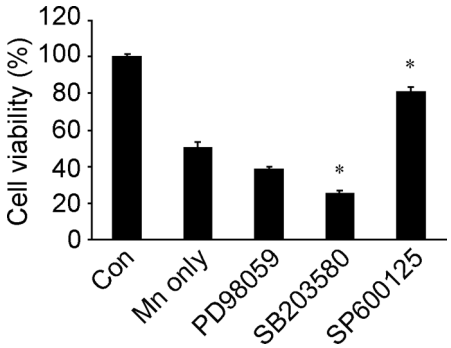

Fig. 2. Effects of MAP Kinase Inhibitors on Toxicity Induced by $MnCl_2$

SN4741 cells were pretreated, each with 10 μM of various MAP kinase inhibitors for 1 hr and incubated for 18 hr with $MnCl_2$ (500 μM). Cytotoxicity was assessed using the XTT assay as described in Materials and Methods. Con, untreated control; Mn only, Mn treatment without MAP kinase inhibitors; PD98059, ERK1/2 inhibitor; SB203580, p38 inhibitor; SP600125, JNK inhibitor. Results are expressed as mean ± SEM of three different experiments. $^*p < 0.05$.

p38 and JNK expression induced by $MnCl_2$ was time dependent (Fig. 1). Maximum activation of both p38 and JNK occurred within 8 hr of the addition of $MnCl_2$.

The activation of p38 was detected as early as 1 hr after $MnCl_2$ treatment and remained elevated even 18 hr after treatment relative to untreated control cells. $MnCl_2$ 500 μM induced the phosphorylation of JNK beginning approximately 4 hr after initiating treatment and remained above this level for at least 24 hr. In contrast to p38 and JNK, $MnCl_2$ treatment did not activate ERK1/2 in SN4741 cells. Furthermore, ERK1/2 fell below the baseline after 12 hr, when p38 and JNK were activated (Fig. 1).

To investigate the role of MAP kinase pathways in Mn-induced DA cell death, we examined the effects of individual specific inhibitors of MAP kinases. $MnCl_2$ (500 μM) induced approximately 50% cell loss after 18 hr treatment (Fig. 2). As shown in Fig. 2, inhibition of ERK1/2 by PD98059 had little effect on cell viability. Interestingly, inhibition of p38 by SB203580 more dramatically reduced the cell viability. However, the number of SN4741 cells was significantly increased when $MnCl_2$ was added together with the JNK inhibitor SP600125.

Although abundant studies suggested that p38 is involved in apoptosis stimulus dependently in some cell types, p38 signaling has been shown to enhance survival, cell growth, and differentiation in different cell lines.[21] We detected the synergistic effects of the specific p38 inhibitor SB203580 on Mn-induced cell death in SN4741 cells (Fig. 2). The family members of p38 kinase are p38α, p38β, p38γ, and p38δ. The p38γ is expressed only in skeletal muscle, whereas p38δ is expressed in the lung, pancreas, kidney, testis, and small intestine.[16,21] In

contrast, p38α and p38β are expressed in most tissues, including the brain and neuronal cells. Thus we examined the effects of the overexpression of p38α and p38β on Mn-induced DA neuronal cell death. As shown in Fig. 3, overexpression of p38α significantly prevented Mn-induced cell death and augmented cell viability, whereas overexpression of p38β displayed no effects on cell viability. Therefore p38α may function as a suppressor of Mn-induced DA neuronal cell death. To confirm the role of p38 isoforms during Mn-induced substantia nigra DA cell death, we used siRNA to suppress p38α and p38β, respectively, as described previously.[18] As expected, when the p38α gene was silenced, Mn-induced substantia nigra DA cell death was enhanced, while p38β siRNA had no effect (Fig. 3).

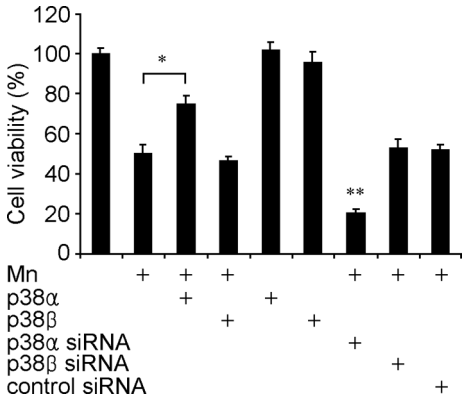

Fig. 3. Involvement of p38α in Preventing DA Neuronal Cell Death

SN4741 cells were transfected with pcDNA3-p38α, pcDNA3-p38β, or empty control vector pcDNA3 for 24 hr and then treated with or without $MnCl_2$ (500 μM). To confirm the p38 isoform-specific role in Mn-induced DA cell death, SN4741 cells were transfected with siRNA specific for the two p38 MAPK isoforms and the control (scrambled) siRNA and then exposed to $MnCl_2$. After 24 hr, cell viability was analyzed using the XTT assay. Results are expressed as mean ± SEM from three different experiments. $^*p < 0.05$, compared with Mn alone; $^*p < 0.01$, compared with untreated control.

We then examined whether expression of p38α was able to affect the activity of caspase-3, a representative apoptotic molecule. In SN4741 cells, overexpression of p38α significantly attenuated the caspase-3 activity induced by $MnCl_2$ (Fig. 4). However, overexpression of JNK1 augmented caspase-3 activity. These findings are consistent with the results in Fig. 2 showing that p38 inhibition and JNK inhibition showed adverse effects in the DA cell death induced by $MnCl_2$.

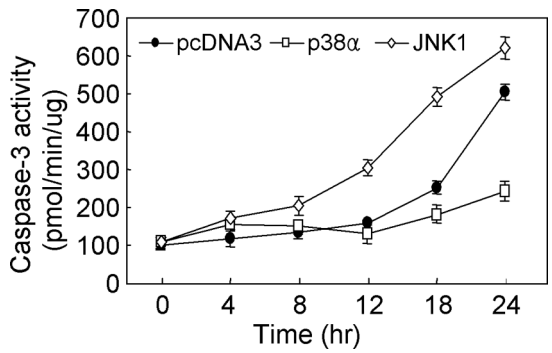

Fig. 4. Effects of p38α and JNK1 on Mn-Induced Caspase-3 Activity in DA Neurons

SN4741 cells transfected with p38α or JNK1 expression vector or empty pcDNA3 vector were treated with $MnCl_2$ (500 μM) for the indicated times. Caspase-3 activity was assayed by cleavage of Ac-DEVD-pNA substrate. Values represent mean ± SEM of triplicate measurements from two independent experiments.

DISCUSSION

The symptoms of Mn-induced neurotoxicity are known to resemble those of PD.[3] Mn has been found to enhance reactive oxygen species (ROS), disrupt mitochondrial energy production, and alter calcium homeostasis.[1, 8, 9] Also, in our previous studies Mn produced a distinct DA cell death profile, which was accompanied by alteration of endoplasmic reticulum (ER) function.[19] On the basis of those observations, recent reports have assumed that various signaling pathways are activated by Mn.[13] However, the exact mechanisms of action of Mn neurotoxicity and the role of MAP kinase family members stimulated by Mn in DA neurons are not clear.

We investigated the precise role of MAP kinase family members stimulated by Mn in DA neurons. The role of MAP kinases in neurodegenerative processes is controversial. While the ERK MAP kinase is mainly activated by receptor tyrosine kinases and generally involved in the control of cell proliferation and neuronal survival pathway, JNK and p38 MAP kinases are preferentially activated by various cellular stresses.[21, 22] Whereas JNK MAP kinases have been shown to be involved in neuronal apoptosis, the role of p38 MAP kinases in neurons is not fully understood. Although the activation of MAP kinase subfamilies by various environmental stresses has been observed in other cells including PC12 cells,[12, 13] there is no information on the signaling pathways involved in the Mn-induced response in substantia nigra DA neurons.

Our results demonstrated that the inhibition

of ERK signaling by PD98059 produced minor changes in the viability of Mn-treated SN4741 cells. On the other hand, JNK inhibition significantly attenuated the Mn-induced substantia nigra DA cell death (Fig. 2). These results are consistent with the general hypothesis that JNK induces apoptosis.[17, 20] Interestingly, inhibition of p38 MAPK more dramatically elevated the Mn-induced substantia nigra DA cell death. Interestingly, we found that p38α, but not p38β, plays a role in preventing Mn-induced substantia nigra DA cell death (Fig. 3). These results suggest that the p38 signaling pathway is not directly involved in Mn-induced cytotoxicity, although the regulation of p38 MAP kinase activation affects cell viability in substantia nigra DA cells. It has been reported that p38α induces apoptosis, whereas p38β has an antiapoptotic function in Jurkat and HeLa cells.[23] Also, p38α induced apoptosis while p38β promoted a hypertrophic response in cardiac myocytes.[24] However, deletion of p38α led to embryonic lethality in mice.[25] These contradictary results indicate that the activation of the p38 pathway occurs in a cell type-specific manner and the various p38 isoforms participate in separate cellular processes. Previous studies suggested that p38 MAP kinase regulates various transcription factors such as MAP kinase-activated protein kinases 2/3, CHOP, ATF-2, and CREB to control numerous cellular responses.[16, 18, 21, 22] Therefore further studies of transcriptional gene regulation by p38 isoforms are required to explain fully the mechanism by which the p38 pathway is activated by Mn in substantia nigra DA neurons.

In conclusion, our results strongly suggest that p38α promotes viability, while JNK mediates cell death in response to Mn toxicity in substantia nigra DA neurons.

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