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Activation of early signaling transcription factor, NF- κ B following low-level manganese exposure

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Abstract

Occupational and environmental exposure to manganese (Mn^{2+}) is an increasing problem. It manifests neuronal degeneration characterized by dyskinesia resembling Parkinson's disease. The study was performed to test the hypotheses whether exposure to Mn^{2+} alters cellular physiology and promotes intracellular signaling mechanism in dopaminergic neuronal cell line. Since transcription factors have been shown to play an essential role in the control of cellular proliferation and survival, catecholaminergic rich pheochromocytoma (PC12) cells were used to measure changes in the DNA binding activities of nuclear factor kappa B (NF- κ B) by electrophoretic mobility shift assay (EMSA) following Mn^{2+} (0.1–10 μ M) exposure. Cells that were exposed to Mn^{2+} produced five-fold-activation of transcription factor NF- κ B DNA binding activity. This remarkable increase was seen within 30–60 min period of Mn^{2+} exposure. Activation of NF- κ B DNA binding activity by Mn^{2+} at 1.0 μ M correlated with proteolytic degradation of the inhibitory subunit I κ B α as evidenced in cytosol. Additional experiments on NF- κ B reporter gene assay also showed increased NF- κ B gene expression at 1.0 and 5.0 μ M Mn^{2+} and this was completely blocked in the presence of NF- κ B translocation inhibitor, I κ B α -DN supporting that NF- κ B induction occurred during Mn^{2+} exposure. In addition, Mn^{2+} exposure to PC 12 cells led to activation of signal responsive mitogen activated protein kinase (MAPKK). These results suggest that Mn^{2+} at a low dose appears to induce the expression of immediate early gene, NF- κ B through MAPKK by a mechanism in which I κ B α phosphorylation may be involved.

Keywords

Mn^{2+} ; NF- κ B; PC12 cells; Oxidative stress; Neurotoxicity

1. Introduction

Manganese (Mn^{2+}) is an essential trace metal required for brain development and functioning of the brain (Prohaska, 1987). On the other hand, Mn^{2+} intoxication in humans is identified with prolonged occupational exposure to high level of this metal. It alters dopaminergic functions specifically in the basal ganglia and produces Parkinson-like disorder (Daniels and Abarca, 1991). Behavioral deficits and neurodegeneration are suggested to be features noted in patients with liver failure are typical of patients with Mn^{2+} neurotoxicity (Pomeir-Layragues et al., 1998; Malecki et al., 1999). Mn^{2+} stimulates DA autoxidation in the dopaminergic

neurons, a processes accompanied by an increase in the formation of quinones (Florence and Stauber, 1989; Shen and Dryhurst, 1998) and protein bound cysteinyl DA and cysteinyl dihydroxyphenylacetic acid (DO-PAC; Hastings et al., 1996). Mn^{2+} uptake, particularly in the globus pallidus, appears to be necessary for the selective vulnerability during Mn^{2+} exposure. This region (among others) is known to be sensitive to energy deprivation and excitotoxic injury. Administration of Mn^{2+} into the rat striatum induced a selective vulnerability of globus pallidus associated with decreased DA content (Lista et al., 1986). Mn^{2+} accumulates within the mitochondria (Gunter et al., 1975) preferentially via the calcium uniporter (Gavin et al., 1990, 1999) and thereby collapsing the mitochondrial membrane potential and producing bio-energetic defect and DNA fragmentation and associated microtubule protein MAP-2 (Malecki, 2001). Apoptosis or necrosis induced by Mn^{2+} may be related to oxidative stress through oxidizing DNA, lipids and proteins (Roth et al., 2000). Like other chemicals Mn^{2+} may induce neurotoxicity by modulating signaling molecules via redox-sensitive transcription factors such as activation protein 1 (AP-1) or nuclear factor kappa-B (NF- κ B) (Baichwal and Baeuerle, 1997; Schrantz et al., 1999). Studies indicate that NF- κ B activation by various chemicals is blocked by antioxidants (Schreck et al., 1991; Schmidt et al., 1995). We hypothesize that activation of these transcription factors would play a significant role in Mn^{2+} induced neurotoxicity that may contribute to cell death. In the present study, we evaluated and characterized the activation of NF- κ B transcription factor and related mitogen activated protein kinase (MAPK kinase) as a target in Mn^{2+} exposure that will help to understand the neuronal degeneration.

2. Methods and materials

2.1. Chemicals

Antibiotics-antimycotics (contains penicillin, streptomycin and amphotericin B), RPMI 1640 medium, and fetal bovine serum were obtained from GIBCO (Grand Island, NY). Glycine, $MnCl_2$, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). $MnCl_2$ was dissolved at 100 mM in water and then all further dilution made in medium. Antibody against I κ B α and double-stranded oligonucleotide having the NF- κ B consensus sequence were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific anti-p44/42 MAP kinase (Thr 202/Tyr 204) antibody was obtained from New England Biolabs, Inc.

2.2. Cell lines

PC12 cells were obtained from the American Tissue and Cell Culture Collection (ATCC, Rockville, MD). Cells were cultured as monolayer in RPMI-1640 medium supplemented with 10% FBS, 5% horse serum, 1% L-glutamine and $1 \times$ antibiotic-antimycotics. Cells were free from mycoplasma as detected by Gen-Probe Mycoplasma Rapid Detection Kit (Fisher Scientific, Pittsburgh, PA). Cells were maintained at 37 °C in an air and 5% CO₂ atmosphere and were used at 4–6 days after passage.

2.3. NF- κ B activation assay

To assay NF- κ B DNA binding activity, electrophoretic mobility shift assay (EMSA) was carried out essentially as described previously (Chaturvedi et al., 1994). Briefly, nuclear extracts (28 μ g protein) prepared from Mn^{2+} treated cells (2×10^6 per ml) were incubated with ³²P end-labeled 45-mer double-stranded NF- κ B oligonucleotide (6 μ g protein with 16 fmoles DNA) from the HIV-LTR, 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3' (bold indicates NF- κ B binding sites) for 15 min at 37 °C, and the DNA-protein complex formed was resolved from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'TTGTTACAAGT-CACTTTCCGCTGCTCACTTTCCAGG-

GAGGCGTGG-3', was used to examine the specificity of binding of NF- κ B to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. The dried gels visualized and 32 P radioactive NF- κ B DNA complex quantitated through Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA) using IMAGE QUANT software.

2.4. Western blot for I κ B α

To examine I κ B α by Western blot, cytoplasmic extracts were prepared from Mn $^{2+}$ -treated and untreated cells and then resolved on 10% SDS-polyacrylamide gels as per the method described earlier (Reddy et al., 1994). After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against I κ B α . The filters were detected by chemiluminescence (ECL, Amersham-Pharmacia Biotech, Arlington Heights, IL).

2.5. Transient transfection and NF- κ B reporter assay

To examine whether Mn $^{2+}$ activates NF- κ B dependant gene expression, we constructed a synthetic NF- κ B-containing promoter element, a PCR-based strategy. The upstream primer (5'-GCGGCCTCGAGGGGACTTTCCCGGG-GACTTTCGGGGACTTTCCGGGACTTTC-CATCCTGCCATCTCAATTAG-3') contained four tandem copies of the NF- κ B binding site (GGGGACTTTCCC) and 18 base pairs of a sequence complementary to the 5' end of the SV40 early promoter sequence, and was flanked with an XhoI site. The downstream primer (5'-GCGGCAAGCTTTTGTCAAAGCCTAGGC-3') was complementary to the 3' end of the SV40 promoter and was flanked with a HindIII site. PCR was performed using the SV40 promoter template. The resulting PCR fragment was digested with XhoI/HindIII and subcloned into a likewise digested SEAP2-promoter plasmid to replace the SV40 minimal promoter element.

PC12 cells were transiently transfected with NF- κ B-SEAP2 (0.5 μ g) and dominant negative I κ B (1 μ M) in addition of empty vector (pCMV) DNA to make up the concentration of DNA 3 μ g for each combination. After transfection (12 h), cells were treated with different concentration of Mn $^{2+}$ for 6 h. Then conditioned medium was removed and assayed for SEAP activity essentially as described by the manufacturer (CLONTECH, Palo Alto, CA). In brief, conditioned medium (25 μ l) was mixed with 30 μ l of five times buffer (500 mM Tris, pH 9, and 0.5% bovine serum albumin) in a total volume of 100 μ l in a 96-well plate and incubated at 65 °C for 30 min. The plate was chilled on ice for 2 min. Then 50 μ l of 1 mM 4-methylumbelliferyl phosphate was added to each well and incubated at 37 °C for 2 h. The activity of SEAP was assayed on a 96-well fluorescent plate reader (Fluoroscan II, Lab Systems, Needham, Heights, MA) with excitation set at 360 nm and emission at 460 nm. The average ($\pm$ S.E.M.) number of relative fluorescent light units for each transfection was then determined and reported as fold activation with respect to control vector-transfected cells.

2.6. Western blot analysis of MAP kinase kinase

The change in oxidative species generation, mitochondrial function and associated cell death in response to various chemical stimuli and other neurotoxicants explain the role in part played by activation of redox sensitive transcription factors, NF- κ B and AP-1 and other related kinases. Since MAPK kinase (mitogen activated protein kinases) is a regulator of NF- κ B transcription factor, the phosphorylated form of MAP Kinase Kinase was assayed in PC 12 cells in response to Mn $^{2+}$ exposure (0–10 μ M) as per the method of Ramesh et al. (1999). Following treatments for 120 min, cell extracts (60 μ g aliquot of total protein) was resolved on each lane on 10% SDS-PAGE, electrotransferred onto nitrocellulose membrane, and probed with the phospho-specific anti-p44/42 MAP kinase (Thr 202/Tyr 204) antibody (New England Biolabs, Inc.) raised in rabbit (1:3000 dilution). The membrane was then incubated with peroxidase-conjugated anti-rabbits IgG (1:3000 dilution), and bands were detected by

chemiluminescence (ECL, Amersham). Same filter was developed for ERK2 monoclonal antibody for loading control.

2.7. Statistics

Data were expressed as mean $\pm$ S.E.M and statistical significance was assessed by using one way analysis of variance followed by Tukeykramer multiple range test. Differences were considered significant at the level of $P < 0.05$.

3. Results

3.1. Mn^{2+} activates NF- κ B transcription factor

EMSA was performed to analyze NF- κ B DNA binding activity in PC12 cells after Mn^{2+} exposure at different dose (0.01–10 μ M) for 120 min. As shown in Fig. 1 Mn^{2+} enhanced NF- κ B DNA binding activity in a concentration dependent manner. The enhancement of NF- κ B DNA binding was about five fold with a maximal stimulation at 0.5–10 μ M Mn^{2+} . The specificity of NF- κ B binding was demonstrated by the disappearance of NF- κ B DNA binding signal with inclusion of the excess unlabelled oligonucleotides.

Super shift assay revealed the specificity of NF- κ B activated during Mn^{2+} exposure. Addition of anti-p50 NF- κ B antibody resulted in the super shift of two NF- κ B binding bands (Fig. 2). Of these, higher band was shifted by the addition of anti-p65 NF- κ B antibody. These suggest that the activated complex of NF- κ B by Mn^{2+} consisted of p50 and p65 subunits. Neither pre immune serum (PIS) nor irrelevant antibodies like anti-cRel or anti-cyclin D1 had any effect on the mobility of NF- κ B. An excess (100-fold) of unlabelled NF- κ B oligo prevented the formation of the band, indicating specificity of NF- κ B binding. To investigate the level of kinetics of NF- κ B activation in Mn^{2+} exposed PC12 cells, cells were treated with 1 μ M Mn^{2+} for various time points (5, 10, 15, 30, 60, 120 min) and nuclear extracts were analyzed for NF- κ B DNA binding. The results in Fig. 3 indicate that Mn^{2+} activated NF- κ B DNA binding in a time dependent manner with a maximum activation at 30 min.

3.2. Mn^{2+} activates degradation of I κ B α

The translocation of NF- κ B to the nucleus is preceded by the phosphorylation and proteolytic degradation of I κ B α in cytosol. To determine the effect of Mn^{2+} on I κ B α degradation, cytosolic extracts were assayed for I κ B α by Western blot analysis. Results obtained (Fig. 4) revealed that Mn^{2+} exposure (1.0 μ M) to PC12 cells caused degradation of I κ B α , within 5 min and completely degrades by 10 min and then reappeared at maximum by 30 min. This disappearance and reappearance of I κ B α corresponds with the kinetics of NF- κ B activation (Fig. 3). The alteration of NF- κ B activation in respect to concentration of Mn^{2+} exposure will help in understanding the mechanisms underlying Mn^{2+} -induced toxicity.

3.3. Mn^{2+} -induced NF- κ B reporter gene expression

Since we have observed that Mn^{2+} activates NF- κ B DNA binding in time and dose dependent manner, further we wanted to examine whether Mn^{2+} activates NF- κ B-dependent gene expression. To determine this, PC12 cells were transfected with a plasmid consisting of gene promoter containing NF- κ B binding sites ligated to the SEAP2 reporter gene. After transfection (12 h), the cells were treated with different concentration of Mn^{2+} for 6 h. and then assayed for SEAP activity. As shown in Fig. 5, there were 2–3-fold increases in SEAP activity in response to 1 and 5 μ M Mn^{2+} , respectively. This was completely blocked in the presence of NF- κ B translocation inhibitor, I κ B α -DN. These results show that Mn^{2+} activates NF- κ B dependent reporter gene expression.

3.4. Mn^{2+} -induced activation of MAPKK

To examine the gene regulation pathways leading to cell death induced by manganese, MAPK kinase were analyzed by Western blot in PC12 cells. Cells that were exposed to various concentrations of Mn^{2+} (0–10 μM) phosphorylated MAPK into 44 and 42 kDa dose dependently as shown in the Fig. 6. In a similar time course, NF- κB was also activated with same dose of Mn^{2+} exposure as shown in Fig. 3. The dose dependent activation of MAPKK suggests that Mn^{2+} activates NF- κB may be through signal responsive kinase.

4. Discussion

Miners exposed to high level of Mn^{2+} are associated with extrapyramidal symptoms (Barbeau, 1984; Aschner, 2000). Potential risks of Mn^{2+} exposure to public arise in the form of gasoline additive methylcyclopentadienyl manganese tricarbonyl (MMT). Mn^{2+} known to induce cell toxicity in which oxidative stress plays an important role (Sun et al., 1993). However, the toxic potential of Mn^{2+} and the exact mechanism by which Mn^{2+} leads to cell death remains elusive.

Several lines of evidence indicate that Mn^{2+} accumulates (~100 nm) within the mitochondria (Maynard and Cotzias, 1955; Gavin et al., 1992; Malecki et al., 1999) and thereby collapses the mitochondrial membrane potential and produces bio-energetic defect impairing oxidative phosphorylation and decreasing ATP synthesis (Brouillet et al., 1993). The decreased cellular protective mechanisms like glutathione and glutathione peroxidase and consequent redox homeostasis loss have been reported in the affected brain regions (Desole et al., 1997; Rabinovich and Hastings, 1998). Our study showed that Mn^{2+} toxicity at the level of 0.01–10 μM in PC 12 cells is associated with activation of nuclear transcription factor, NF- κB . Mn^{2+} is known to disturb the mitochondrial respiration and inhibits the antioxidants system (Maynard and Cotzias, 1955; Brouillet et al., 1993) and consequently straining the cell's ability to combat oxidative stress mediated effect. NF- κB is activated in many different cell types following challenge with several pathogenic stimuli (Gilmore, 1990). Though most, if not all, inducers of NF- κB seem to relay on the production of reactive oxygen intermediates (Meyer et al., 1993), we observed non-significant generation of oxidative species (data not shown) in Mn^{2+} -induced NF- κB activation. Mn^{2+} dose for optimal NF- κB activation though, was not the same as that for optimal oxidative species production, it is suggested that oxidative radicals produced in response to Mn^{2+} might be necessary for NF- κB activation.

NF- κB is present in the cytosol as an inducible multi subunit complex of 50 kDa (p50) and 65 kDa (p65) polypeptide complexed with an inhibitor subunit I $\kappa B\alpha$. The inhibitory protein, I $\kappa B\alpha$, tightly controls NF- κB . Upon activation, I $\kappa B\alpha$ dissociates from NF- κB heterodimer by undergoing sequential phosphorylation and degradation, thereby release NF- κB for nuclear translocation and facilitate DNA binding and transcriptional up-regulation of genes down stream of κB motif. Mn^{2+} -induced NF- κB activation in the present study was accompanied by characteristic phosphorylation and degradation of I $\kappa B\alpha$ in a time-dependant way. In Mn^{2+} exposed PC12 cells, the disappearance and reappearance of I $\kappa B\alpha$ subunit in the cytosolic fraction at the time point in which NF- κB nuclear translocation occurred was well correlated. The studies with monoclonal antibodies directed to p50 and p65 indicated that Mn^{2+} -induced NF- κB complex comprises p50 and p65 heterodimers. To examine whether NF- κB plays a role in signaling, we assayed reported gene expression. Treatment with Mn^{2+} resulted in 2–3-fold increase of NF- κB -induced SEAP2 expression. This effect was blocked by simultaneous treatment with I $\kappa B\alpha$ -DN (NF- κB translocation inhibitor). This reflects the induction of NF- κB during Mn^{2+} exposure in the dopaminergic cell death. Though the significant role of NF- κB in Mn^{2+} -induced cell death mechanism is not known, previous reports indicate that (Baichwal and Baeuerle, 1997; Ishikawa et al., 1997) activation of NF- κB transcription factor could lead downstream to excessive transcription of proteins that contribute to cell death. Further, increased oxidative production is also seen in with many other NF- κB stimuli, which

was blocked by antioxidants (Schreck et al., 1991; Schmidt et al., 1995). In contrast, in some other cell lines, it was shown that TNF- α , a potent stimulator of NF- κ B nuclear translocation prevented cell death (Cheng et al., 1994).

We have previously reported that heavy metals like lead activates NF- κ B through MAPKK activation (Ramesh et al., 1999) in PC12 cells. The mechanism by which the NF- κ B induction during Mn²⁺ exposure may be related to MAPKK activation as reported in other studies (Ramesh et al., 1999; Lee et al., 1998). The phosphorylation of I κ B α is regulated by a large number of kinases, including IKK- α , IKK- β , and mitogen activated protein/extracellular signal-related kinase kinases (Karin, 1999). The I κ B α activation and phosphorylation by MAPKK has been shown in both in vitro and in vivo models (Lee et al., 1997, 1998). In PC 12 cells, the degradation of I κ B α during Mn²⁺ exposure may be due to the activation of either of these kinase regulators. Dose dependent activation of NF- κ B regulator MAPKK following Mn²⁺ in our study further suggesting that MAPKK may influence the activation of NF- κ B transcription factor possibly through either IKK- α or IKK- β in the mechanism of Mn²⁺-induced toxicity.

In summary, although the contribution of oxidative stress to NF- κ B activation is speculative, there appears to be ample evidence suggesting that Mn²⁺-induced oxidative species may contribute to cell death by multiple mechanisms involving NF- κ B activation. Further we suggest that MAPKK activation may initiate NF- κ B, which might be involved in the dopaminergic toxicity during Mn²⁺ exposure. The functions of NF- κ B in cell death and the mechanisms of its regulation by oxidative stress in Mn²⁺-induced dopaminergic toxicity deserve further study. This finding is important because targeting NF- κ B is a potential mechanistic pathway in both environmental and idiopathic forms of PD.

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Fig. 1.

Dose dependent activation of NF- κ B DNA binding by Mn^{2+} . Cells (2×10^6 cells per ml) were incubated with different concentrations of Mn^{2+} (0–10 μM) for 120 min and then nuclear extracts were prepared and activation was determined by electrophoretic mobility shift assay as described in Section 2. Units at the bottom indicate fold increase in Mn^{2+} -induced NF- κ B activation with dose compared with untreated control cells. Similar results were obtained in three independent experiments.

Fig. 2.

Super shift and specificity assay revealed Mn^{2+} -induced activation of NF- κ B consists of p50 and p65 sub units complex. Treated and untreated nuclear extracts were incubated with different antibodies and 50 times excess cold probe for 30 min at 37 °C and then assayed by EMSA with labeled wild NF- κ B probe as described in Section 2.

Fig. 3.

Time dependent activation of NF-κB DNA binding by Mn^{2+} in PC12 cells. Cells (2×10^6 cells per ml) were exposed to Mn^{2+} ($1 \mu\text{M}$) at various time points as indicated and then activation was determined in the nuclear extracts by Electrophoretic mobility shift assay as described in the Section 2. Units at the bottom indicate fold increase in Mn^{2+} -induced NF-κB activation with time compared with untreated control cells. Similar results were obtained in three independent experiments.

Fig. 4. Mn^{2+} -induced $\text{IkB}\alpha$ degradation in the cytosolic fraction. Cells (2×10^6 cells per ml) were incubated with Mn^{2+} (1 μM) at different time periods (0–120 min) and detected $\text{IkB}\alpha$ in the cytosolic fraction by Western blot using polyclonal antibody against $\text{IkB}\alpha$ as described in the Section 2. Degradation of $\text{IkB}\alpha$ is apparent at 10 min and reappeared at 30 min.

Fig. 5. Mn^{2+} -induced NF- κ B reporter gene expression in PC12 cells. Cells were transfected with a plasmid consisting of gene promoter containing NF- κ B binding sites ligated to SEAP2 reporter gene and then cells were treated with different concentration of Mn^{2+} for 6 h. and assayed for SEAP activity as described in the Section 2. After treatment significant increase of SEAP2 expression is observed at ($P < 0.001$).

Fig. 6. Mn^{2+} -induced activation of MAPKK in PC-12 cells. Cells were exposed to various concentrations of Mn^{2+} (0–10 μM) and assayed for MAPKK activation by Western blot using antibody specific to phospho-specific p44/42 MAP kinase as described in the Section 2. Dose dependent activation of MAPKK was observed following Mn^{2+} exposure. Same filter was developed for ERK2 monoclonal antibody for loading control. Similar results were obtained in three independent experiments.