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Manganese regulates caspase-3 gene promoter activity by inducing Sp1 phosphorylation in PC12 cells

Atsuhiro Uchida[a], Kentaro Oh-hashi[b], Kazutoshi Kiuchi[a,b], Yoko Hirata[a,b,*]

[a] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu 501-1194, Japan
[b] Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu 501-1193, Japan

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ABSTRACT

Chronic manganese exposure causes selective toxicity to the dopaminergic system, resulting in a parkisonian-like neurological condition known as manganism. Manganese causes a typical apoptosis, which includes activation of the caspase cascade and DNA fragmentation in PC12 cells. Caspase-3 is a major contributor to the execution of neuronal apoptosis. In a previous study, we demonstrated that caspase-3 cleavage and expression of pro-caspase-3 mRNA and protein increased in PC12 cells treated with manganese, but this response was not observed with other apoptosis inducers. To understand the molecular mechanisms that regulate expression of caspase-3 in manganese-treated PC12 cells, we characterized the 5′-flanking region of the rat caspase-3 gene and identified both a core promoter and a manganese-responsive region that contains three putative Sp1 binding sites. Furthermore, manganese treatment induced robust Sp1 phosphorylation and increased its DNA binding activity. Overexpression of mutant Sp1 lacking phosphorylation sites attenuated Sp1's ability to stimulate manganese-induced caspase-3 promoter activity. In conclusion, our results indicate that Sp1 phosphorylation is required for manganese-induced transactivation of caspase-3.

1. Introduction

Manganese is a trace metal that is essential for many enzymes important in normal cellular functions, including manganese superoxide dismutase, phosphoenolpyruvate decarboxylase, and glutamine synthetase (Prohaska, 1987; Takeda, 2003). However, manganese is toxic at high concentrations. Accumulation of excess manganese in the brain results in manganism, a neurological syndrome with cognitive, psychiatric, and movement abnormalities similar to Parkinson's disease. The adverse effects of inorganic manganese dust or vapor among steel manufacturing workers or welders are well known, as well as the health risks of exposure to organic manganese compounds, such as the pesticide manganese ethylenebis (Thiruchelvam et al., 2000; Zhang et al., 2003) and an antiknock agent found in unleaded gasoline, methylcyclopentadienyl manganese tricarbonyl (Kitazawa et al., 2002). Although a very limited number of autopsy studies have been performed on persons who were exposed to manganese, pathological evidence suggests that pallidum is damaged in manganese encephalopathy (Yamada et al., 1986; Perl and Olanow, 2007), indicating that some relationship exists between manganism and Parkinson's disease. Parkinson's disease is the second-most common neurodegenerative disorder and is characterized by resting tremors, akinesia/bradykinesia, rigidity, and postural instability due to the selective loss of dopaminergic neurons in the substantia nigra.

Manganese is selectively toxic to dopaminergic neurons in experimental animals. Dopamine levels decrease in the basal ganglia of monkeys exposed to manganese via inhalation (Bird et al., 1984; Eriksson et al., 1992) and in the rat striatum upon direct injection of manganese (Lista et al., 1986; Brouillet et al., 1993). However, despite several previous studies, the mechanism underlying manganese-induced neurotoxicity remains unclear. Accumulating evidence suggests that manganese induces apoptosis (i.e., programmed cell death), in various cell lines, including rat adrenal pheochromocytoma PC12 cells, human cervical carcinoma HeLa cells, mouse fibroblast NIH3T3 cells, and human B cells (Hirata et al., 1998a; Schrantz et al., 1999; Oubrahim et al., 2001, 2002). Upon induction of apoptosis, manganese activates various intracellular signaling pathways, including c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, extracellular signal

Abbreviations: ECL, enhanced chemiluminescence; EMSA, electrophoresis mobility shift assay; RLA, relative luciferase activity; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

* Corresponding author at: United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan. Tel.: +81 58 293 2609; fax: +81 58 293 2794.
E-mail address: yokoh@gifu-u.ac.jp (Y. Hirata).

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regulated kinase, and p70 S6 kinase in cultured cells (Hirata et al., 1998a, 1998b; Walowitz and Roth, 1999; Hirata, 2002). Manganese also activates the mitochondrial and endoplasmic reticulum stress-mediated apoptotic pathways (Chun et al., 2001; Oubrahim et al., 2002). Both apoptotic signals are integrated downstream to induce caspase-3 activity.

Caspase-3 is a primary mediator of apoptosis. It initially exists as pro-caspase-3 (32 kDa), which is activated by proteolytic cleavage at Asp-28/Ser-29 (between the N-terminal prodomain) and at Asp-175/Ser-176 (between the large and small subunits) to generate a large subunit of 17 kDa and a small subunit of 12 kDa (Riedl and Shi, 2004). In response to apoptotic stimuli, the amount of pro-caspase-3 generally decreases as caspase-3 cleavage increases. However, it has been shown that mRNA and protein levels of caspase-3 are elevated during neuronal apoptosis in vivo (Yakovlev et al., 1997; Chen et al., 1998; Harrison et al., 2000). Furthermore, we have previously shown that manganese up-regulates both caspase-3 mRNA and protein and increases caspase-3 cleavage in cultured cells (Ito et al., 2006). Taken together, these results suggest that increases in caspase-3 expression promote apoptosis. However, the mechanism by which manganese induces increases in caspase-3 expression remains unclear. In this study, we analyzed the effect of manganese on caspase-3 gene promoter activity and the role of Sp1 in manganese-induced expression of caspase-3.

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma–Aldrich (MO, USA) unless otherwise indicated. Mouse monoclonal anti-Sp1 (1C6) and anti-α-tubulin (TU-02) antibodies were obtained from Santa Cruz Biotechnology (CA, USA). Rabbit polyclonal anti-Sp1 (phospho T453) (ab59257) was from Abcam plc (Cambridge, UK).

2.2. Construction of luciferase reporter vectors

Rat caspase-3 promoter (−1815/+45 corresponding to 73–1932 of AF427079) was cloned into the NheI and XhoI restriction sites of pGL3-Basic (Promega Corporation, WI, USA). Progressive deletion constructs of the caspase gene promoter were engineered by unidirectional cloning of PCR fragments from the promoter between the NheI and XhoI sites of pGL3-Basic. The promoter region was defined using a database of the NIH MGC Project (Strausberg et al., 2002) (Fig. 1). Thus, the transcription start site (+1) corresponded to 1888 of AF427079, which is 57 bases downstream of the transcription start site described by Liu et al. (2002).

2.3. Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (Wako Pure Chemicals, Osaka, Japan) supplemented with 7% horse serum (Invitrogen Corporation, CA, USA) and 4% fetal bovine serum (Equitech-Bio, Inc., TX, USA) at 37 °C in 5% CO_2.

2.4. Transfection and luciferase assay

PC12 cells seeded into a 24-well plate (5×10^5 cells/well) were cultured for 48 h and transfected with reporter constructs and pGL4.70[hRluc] as an internal standard using the TransFast™ transfection reagent (Promega Corporation). Twenty-four hours after transfection, the cells were treated with manganese at the indicated concentrations for 18 h. Luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega Corporation) in accordance with the manufacturer's instructions. Briefly, the cells were lysed in 100 μl of Passive Lysis Buffer (Promega Corporation) for 15 min at 25 °C. The lysates were centrifuged at 15,000 × *g* for 5 min, and 5 μl of the supernatant was mixed with 25 μl of firefly luciferase assay reagent, followed by 25 μl of *Renilla* luciferase assay reagent. Luminescence intensity was measured using a GloMax™ 20/20n Luminometer (Promega Corporation).

2.5. Preparation of nuclear extracts

PC12 cells were grown in a 10-cm dish. The cells (approximately 1.2×10^7 cells) were resuspended in 10 volumes of lysis buffer (250 mM sucrose, 20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 1 μg/ml leupeptin) and homogenized in a glass Teflon-homogenizer (20 strokes, 800 rpm). The homogenate was centrifuged at 800 × *g* for 10 min at 4 °C. The pellets were washed with lysis buffer and resuspended in an equal volume of nuclear extraction buffer (25% glycerol, 420 mM NaCl, 20 mM HEPES–KOH (pH 7.5), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The nuclear suspensions were centrifuged at 15,000 × *g* for 15 min at 4 °C. The nuclear extracts were diluted with nuclear extraction buffer to normalize protein concentrations for each sample.

2.6. Western blotting

Samples were homogenized in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.1% bromophenol blue) by sonication. The protein concentration in each preparation was measured with a DC Protein Assay kit (Bio-Rad Laboratories, CA, USA) using γ-globulin as a standard. β-Mercaptoethanol (5%) was added, and the mixture was boiled for 5 min. Aliquots containing 40 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond-enhanced chemiluminescence (ECL), GE Healthcare UK Ltd., Buckinghamshire, England). Membranes were blocked with blocking buffer (5% non-fat dry milk in phosphate-buffered saline with 0.05% Tween20 (PBST) for 1 h and then incubated with the blocking buffer containing anti-Sp1 (1:500), anti-Sp1 (phospho T453; 1:500) or anti-α-tubulin (1:1000). The blots were washed and incubated with secondary antibodies conjugated to horseradish peroxidase (1:2000, Cell Signaling Technology, MA, USA). Immunoreactive bands were visualized with ECL (GE Healthcare UK Ltd.) or Super-Signal West Dura (Thermo Fisher Scientific, MA, USA). In some cases, blots were reprobed with different antibodies after stripping at 50 °C for 30 min in 62.5 mM Tris–HCl (pH 6.7), 100 mM β-mercaptoethanol, and 2% SDS.

2.7. Electrophoresis mobility shift assay (EMSA)

EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Nuclear extracts (5 μg of protein) were prepared as previously described and then pre-incubated in 50 μl of binding buffer (10 mM Tris–HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 3 μg of salmon sperm DNA, and 5% glycerol) for 30 min at 25 °C. Subsequently, the reaction was further incubated with biotin end-labeled 39-mer double stranded Sp1 oligonucleotides (20 nM) from the caspase-3 gene promoter (5′-GAG**GGGGCGGGGC**G**TGGGCGGGGC**G**TGGGCGGGA**CCGGC-3′; bold indicates Sp1 binding sites) for 30 min at 25 °C. The resultant DNA-protein complex was resolved from free oligonucleotide in a 4% native polyacrylamide gel in Tris borate–EDTA buffer, and transferred to nylon membranes (Biodyne, Pall Corporation, MI, USA). The membranes were cross-linked by UV radiation using a GS Gene-Linker (Bio-Rad Laboratories). Biotin signals were detected using a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. A double-stranded mutated oligonucleotide (5′-GAGGG**TT**CG**TTT**CGTG**TT**CG**TTT**CGTG**TT**C**TTT**ACCGGC-3′) and an unlabeled double stranded Sp1 oligonucleotides were used to examine the specificity of DNA binding for Sp1.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression levels were analyzed via RT-PCR, and total RNA was isolated using TRIZOL (Invitrogen Corporation). Equal amounts of RNA (1.5 μg) were reverse-transcribed at 50 °C for 60 min with Superscript reverse transcriptase™ III (Invitrogen Corporation) in a 20 μl volume containing 0.5 μg of oligo(dT)12–18 primer (Invitrogen Corporation); 50 mM Tris–HCl (pH 8.3); 75 mM KCl; 3 mM $MgCl_2$; 40 U RNaseOUT (Invitrogen Corporation); 0.5 mM dATP, dCTP, dGTP and dTTP; and 10 mM dithiothreitol. Reactions were terminated by heating for 15 min at 70 °C. cDNA was amplified in PCR buffer by denaturing for 30 s at 94 °C, annealing for 30 s at 58 °C, and extending for 30 s at 72 °C. This process was performed for 27 and 20 cycles for caspase-3 and β-actin, respectively. The primer sequences were as follows: caspase-3 forward, 5′-ACGGTACGCGAAGAAAAGTGAC-3′ (NM_012922, 4–25), and caspase-3 reverse, 5′-TCCTGACTTCGTATTTCAGGGC-3′ (NM_012922, 264–285), yielding a 282-bp product, and β-actin forward, 5′-TGTATGCCTCTGGTCGTACC-3′ (NM_031144, 425–444), and β-actin reverse, 5′-CAACGTCACACTTCATGATGG-3′ (NM_031144, 842–862), yielding a 438-bp product. The number of cycles selected for each primer pair and the template quantity were determined to be in the linear range for each gene. The PCR products were analyzed in 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. Images were captured with a Gel Print 2000i/VGA and analyzed with a Bio Image Intelligent Quantifier (RMLuton, Inc., MI, USA). The levels of caspase-3 mRNA were measured in arbitrary units and defined as the proportion of caspase-3 PCR product intensity to β-actin PCR product intensity within a single RNA sample that was expressed as percentage change relative to the control.

2.9. Statistical analyses

Numerical data were statistically analyzed with GraphPad Prism 4 (GraphPad Software, Inc., CA, USA). The significance of differences between experimental groups was determined by Bonferroni's multiple comparison tests in conjunction with ANOVA. When two groups were compared, Student's *t*-test was used; $p < 0.05$ was considered statistically significant.