Anti-apoptotic and pro-apoptotic effect of NEPP11 on manganese-induced apoptosis and JNK pathway activation in PC12 cells

Yoko Hirata[1], Kyoji Furuta[2], Satoshi Miyazaki[1], Masaaki Suzuki[2], Kazutoshi Kiuchi[1]

[1]Department of Biomolecular Science, Faculty of Engineering, Gifu University, [2]Regeneration and Advanced Medical Science, Graduate School of Medicine, Gifu University, Gifu 501-1193, Japan

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Corresponding author:
Dr. Yoko Hirata
Department of Biomolecular Science, Faculty of Engineering, Gifu University
1-1 Yanagido, Gifu 501-1193, Japan
Phone:+81-58-293-2609
Fax:+81-58-230-1893
E-mail: yoko@biomol.gifu-u.ac.jp

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Abstract

Neurite outgrowth-promoting prostaglandins (NEPPs), cyclopentenone prostaglandin derivatives, are found to be neurotrophic. These small organic compounds promote neurite outgrowth of PC12 cells and dorsal root ganglion explants in the presence of nerve growth factor, and prevent neuronal cell death of HT22 cells and cortical neurons induced by various stimuli. In this study, we examined whether NEPP11 prevents manganese-induced apoptosis of PC12 cells. NEPP11 (5 μM) attenuated manganese-induced DNA fragmentation by approximately 50%. In addition, NEPP11 partially prevented manganese-induced c-Jun phosphorylation and c-Jun N-terminal kinase (JNK) phosphorylation determined by Western blotting. Inhibition of the JNK signaling pathway by NEPP11 appeared to be selective, because NEPP11 did not inhibit manganese-induced activation of p38 mitogen activated protein kinase (p38 MAPK), extracellular signal-regulated kinase1/2 (ERK1/2), MEK1/2, and p70 S6 kinase (p70S6K) in PC12 cells. In contrast, NEPP11 alone was toxic at higher concentrations (>10 μM) producing DNA fragmentation and activation of the JNK pathway. Molecular modifications of NEPP11 may strengthen its inhibitory effects on the JNK pathway while preventing its cytotoxicity, and thus may become a useful small molecule reagent for the treatment of manganese toxicity and other similar neurodegenerative processes.

Theme: DISORDERS OF THE NERVOUS SYSTEM

Topic: Neurotoxicity

Keywords: cyclopentenone prostaglandins, DNA fragmentation, JNK, manganese, PC12 cells, SP600125

1. Introduction

The development of a small organic molecule that mimics or enhances the action of neurotrophic factors may provide therapeutic agents for neurodegenerative disease. In this context, cyclopentenone prostaglandins (PGs) that are found to function as neurite outgrowth-promoting prostaglandins (NEPPs) are potential candidates. NEPPs, derivatives of Δ^7-PGA_1 methyl ester, are efficiently synthesized by three-component chemical synthesis (5,21). NEPPs promote faster neurite outgrowth from PC12 cells and from dorsal root ganglia in the presence of nerve growth factor (NGF) (26). In addition, NEPPs protect HT22 cells, a mouse hippocampal cell line, against oxidative glutamate toxicity and cortical neurons against glutamate, NO donor, and serum deprivation induced cell death (27). Most importantly, NEPP11, the most effective compound, protects the brain in focal ischemia model of mice with permanent middle cerebral artery occlusion by intraventricular administration, suggesting that NEPP11 has neuronal survival-promoting activity in vivo (27).

Chronic exposure to manganese is known to cause neurological symptoms similar to idiopathic Parkinson's disease in both human and laboratory animals (2). For example, occupational exposure during mining has been shown to cause a Parkinson's like syndrome known as Manganism (7). Although the effects of long-term environmental exposure to

manganese are uncertain, manganese and certain pesticides that inhibit mitochondrial function have emerged as a putative risk factor of Parkinson's disease (6,7,20), where apoptosis has been suggested to play a role in the death of cells (15). Manganese induces apoptosis in dopamine-producing rat pheochromocytoma (PC12) cells (4,11,24), human B cells (28) and HeLa cells (22). In the present study, a PC12 cell model system was used to assess the neuroprotective effect of NEPP11 (Fig. 1) against manganese toxicity. The results show that NEPP11 has a biphasic effect on manganese induced apoptosis in PC12 cells, as well as JNK, c-Jun pathway activation. Since the concentration ranges of NEPP11 in both actions match each other very well, it is quite possible that the anti-apoptotic action of NEPP11 is related to its inhibiting function in JNK pathway.

2. Materials and methods

2.1. Materials

c-Jun and p70 S6 kinase (p70S6K) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stress-activated protein kinase (SAPK)/JNK, p44/42 mitogen activated protein kinase (MAPK)/ERK, MEK1/2, p38 MAPK polyclonal antibodies, phospho-c-Jun (Ser73), phospho-p70S6K (Thr389), phospho-p44/42 MAPK/ERK (Thr202/Tyr204), phospho-MEK1/2 (Ser217/221), phospho-p38 MAPK

(Thr180/Tyr182), phospho-MLK3 (Thr277/Ser281) polyclonal antibodies and phospho-SAPK/JNK (Thr183/Tyr185) monoclonal antibody were purchased from New England Biolabs (Beverly, MA, USA). The synthesis of NEPP11 was described previously (5). The compounds were dissolved in ethanol. SP600125 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). All other chemicals used were of analytical grade.

2.2. Cell culture

The rat pheochromocytoma cell line PC12 (8) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% horse serum (HS) (BioWhittaker, Walkersville, MD, USA) and 4% fetal bovine serum (FBS) (Biofluids, Rockville, MD, USA) at 37°C in 5% CO_2.

2.3.Analysis of DNA fragmentation

PC12 cells (~ 2 x 10^7 cells) were incubated at 37°C for the indicated periods in DMEM supplemented with 5% FBS. Cells were resuspended in lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 0.5% Triton X-100) and kept on ice for 15 min. After centrifugation at 14,000 x g for 10 min, the soluble DNA was isolated and extracted with Tris/EDTA (TE)-saturated phenol and phenol/chloroform (1:1), followed by ethanol precipitation, essentially as described previously (14). DNA dissolved in TE buffer was incubated with RNase A (50

μg/ml) at 37°C for 1 h. Approximately half of the recovered soluble DNA per condition was separated by electrophoresis in 1.2% agarose gels and visualized with an ultraviolet transilluminator. Gel photographs were taken with a gel imaging system and quantification of bands was performed using the imaging software, Bio Image Intelligent Quantifier (RMLuton, Jackson, MI, USA). Statistical differences between controls and treated groups were assessed by the two-tailed, unpaired Student's t-test.

2.4. Western blotting

Cells were lysed in sodium dodecyl sulfate (SDS)-Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol) and sonicated for ~ 20 s on ice. The protein concentration was measured by the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) using γ-immunoglobulin as a standard. Total cell lysates (40 μg protein) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked in blocking buffer (phosphate-buffered saline plus 0.05% Tween-20 and 5% non-fat dry milk) for 1 h or more, followed by incubation with the appropriate antibody at 4°C overnight. The membranes were washed at least three times and incubated with HRP-conjugated secondary antibody for 90 min at room temperature (Amersham Biosciences). The protein was visualized with the enhanced chemiluminescence (ECL) system

(Amersham Biosciences). In some cases, blots were reprobed with different antibodies after stripping in 62.5 mM Tris-HCl (pH 6.7)/100 mM β-mercaptoethanol / 2% SDS.

3. Results

3.1. NEPP11 prevents manganese-induced DNA fragmentation

NEPP11 is considered to have neurotrophic properties because it prevents cytotoxicity in cortical neurons resulting from exposure to glutamate, NO donors, and serum deprivation (27). We have previously reported that manganese induces DNA fragmentation, a biochemical hallmark of apoptosis in dopamine-producing PC12 cells (11). Here, we examined whether NEPP11 prevents manganese-induced DNA fragmentation in PC12 cells. NEPP11 (5 μM) significantly reduced manganese-induced DNA fragmentation to approximately 50% (Fig. 2). The inhibitory effect was dose related (21% at 2.5 μM; 12% at 1.25 μM), however, at higher concentrations (> 10 μM) NEPP11 itself induced extensive DNA fragmentation (Fig. 4A). Arachidonic acid precursor (10 μM) has no effect on manganese-induced DNA fragmentation (data not shown). These results suggest that NEPP11 prevents manganese-induced apoptosis within a certain range of concentrations.

3.2. NEPP11 inhibits the phosphorylation of c-Jun and JNK but not MKK4 induced by manganese

To investigate the intracellular mechanism of NEPP11, we examined the effect of NEPP11 on signaling cascades activated by manganese, such as JNK, ERK, p38 MAPK and p70S6K (11-13,30). To clarify which pathway NEPP11 may affect, PC12 cells were preincubated with NEPP11 for 30 min and followed by co-incubation with manganese for another 20 h. Cellular proteins were analyzed by Western blotting. As previously reported, manganese dose-dependently induced the phosphorylation of c-Jun, JNK, p44/42 ERK, p38 MAPK and p70S6K (Fig. 3ABCD). Manganese also increased p44/42 ERK kinase (MEK1/2) phosphorylation. NEPP11 (5 μM) partially blocked manganese-induced JNK activation and subsequent phosphorylation of c-Jun (Fig. 3A). Concentrations of NEPP11 required to inhibit JNK activation were similar to those needed to prevent DNA fragmentation, suggesting that these two activities were related. In contrast, NEPP11 had no effect on c-Jun protein levels, nor did it change the phosphorylation states of p44/42 ERK, MEK1/2, p38 MAPK and p70S6K, (Fig. 3BCD), indicating that NEPP11 may be a specific inhibitor of JNK activation. As shown in Fig. 3A, NEPP11 had no effect on the manganese-induced phosphorylation of SEK1/MKK4, suggesting that NEPP11 does not inhibit the mixed lineage kinases (MLKs), the upstream kinases of SAP1/MKK4 (3,10,23,29).

3.3. NEPP11 at 20 μM is a strong activator of the JNK pathway

To further characterize the molecular mechanisms of NEPP11 we examined the effect of

NEPP11 itself on the JNK pathway. As shown in Fig. 4AB, 20 μM NEPP11 induced extensive DNA fragmentation and robust activation of the JNK pathway. Thus, the phosphorylation of MKK4 (a JNK kinase), JNK and c-Jun was greatly increased. It has recently been reported that MLK3 acts upstream of MKK4 (3,9,10,23,29), which, in turn, lies upstream of JNKs and of c-Jun. Phosphorylation of MLK3 from NEPP11- and manganese-treated PC12 cells was increased compared with untreated controls (Fig. 4B), suggesting that NEPP11 and manganese can modulate MLK3, by affecting autophosphorylation (17). In contrast, the increases of phospho-p44/42 ERK are similar in NEPP11- and manganese-treated PC12 cells. We have shown that manganese is a potent activator of the JNK pathway compared with other apoptosis inducers (11). Here we found that NEPP11 itself was also strong inducer of the JNK pathway. Taken together, our results indicate that NEPP11 has a biphasic effect on the JNK pathway. At low concentrations it inhibits JNK, but at high concentrations it drives the activation of the JNK pathway.

3.4. SP600125 inhibits both manganese induced DNA fragmentation and phosphorylation of c-Jun.

Finally, to evaluate the JNK pathway in manganese toxicity, we examined the effect of the JNK inhibitor SP600125 (1), on manganese-induced DNA fragmentation. SP600125 inhibited both DNA fragmentation (Fig. 5A) and c-Jun phosphorylation induced by

manganese (Fig. 5B). These results further support that the inhibition of the JNK pathway can attenuate manganese-induced apoptosis. SP600125 at 50 μM alone induced DNA fragmentation even in the absence of c-Jun phosphorylation, suggesting that 50 μM SP600125 is toxic but in a manner independent of the JNK pathway. Interestingly, SP600125 enhanced the phosphorylation of ERK in the absence or presence of manganese (Fig. 5B).

4. Discussion

The two major biological functions of NEPPs described to date are the promotion of neurite outgrowth and neuroprotection from apoptotic stimuli (26,27). Levels of the immunoglobulin heavy chain binding protein/glucose-regulated protein 78 (BiP/GRP78) increase in response to NEPPs and overexpression of BiP/GRP78 protein promotes neurite outgrowth induced by NGF (26). These results suggest that BiP/GRP78 is involved in the intracellular mechanism of neurite outgrowth-promotion induced by NEPPs. In contrast, the molecular mechanism underlying the neuroprotective effects of NEPPs remains to be elucidated. It has been reported that NEPPs interact with intracellular proteins not yet identified (27). In the present study, we found that NEPP11 prevented manganese-induced phosphorylation of JNK and c-Jun but not ERKs, p38 MAPK or p70S6K. These results

suggest that the JNK pathway is a specific target of NEPP11. Since NEPP11 did not inhibit the phosphorylation of MKK4, MLK3, an upstream kinase of MKK4, appears not to be a target of NEPP11 and suggests further that MKK4 may be a direct target.

To our knowledge, CEP-1347 (KT7515) and SP600125 have both been reported to inhibit the JNK pathway, however, the targets of their respective inhibitory effects appear to be different (Fig. 6). CEP-1347 (KT7515) is a semisynthetic derivative of the fermentation product K-252a, a broad kinase inhibitor having a neuroprotective effect in various contexts (16). Inhibition of the JNK pathway by CEP-1347 (KT7515) has been shown to suppress motoneuron apoptosis and MPTP-mediated loss of nigrostriatal dopaminergic neurons (18,25). Maroney et al. (19) have found that the direct molecular target of CEP-1347 (KT7515) is the MLK family. CEP-1347 directly inhibits the enzyme activities of the MLK family members *in vitro* and prevents endogenous activation of MLK3, as determined by a reduced electrophoretic mobility in SH-SY5Y cells. On the other hand, SP600125 is a small-molecule inhibitor of JNK *in vitro* and, to a lesser extent, MKK4 (1). SP600125 inhibits both the phosphorylation of c-Jun and JNK in Jurkat T cells, and anti-CD3-induced apoptosis of CD4+ CD8+ thymocytes (1). The present results also show that manganese-induced DNA fragmentation can be attenuated by the treatment with SP600125 at concentrations that blocked manganese-induced c-Jun phosphorylation in PC12 cells.

Although both NEPP11 and SP600125 alone cause DNA fragmentation at high concentrations, NEPP11, but not SP600125 activates the JNK pathway. Interestingly SP600125 alone activates ERK. This may have some relevance for its neurotrophic action, since ERK activation has been suggested to be important for neurotrophic activity (32). Since higher concentrations of NEPPs were toxic and the range of effective concentrations was limited, further modification of NEPPs may provide small molecules that have potential in neurodegenerative disease. The possible therapeutic utility of cyclopentenone prostaglandin A_1 has been also shown in rotenone-induced apoptosis of neuroblastoma cells (31).

The present results support an alternative conclusion that NEPP11 has a biphasic effect on manganese induced apoptosis in PC12 cells, as well as JNK, c-Jun pathway activation. Since the concentration ranges of NEPP11 in both actions match each other very well, it is quite possible that the anti-apoptotic action of NEPP11 is related to its inhibiting function in JNK pathway. Furthermore, data from the study of SP600125, a recently developed JNK inhibitor, confirmed the importance of JNK in manganese-induced apoptosis of PC12 cells. Taken together, these findings support the idea that the JNK pathway inhibitors may prove to have clinical benefits in diseases involving apoptotic cell death, and small trophic molecules which can cross the blood-brain barrier, such as NEPP11, might be an alternative

therapeutic approach in the treatment of neurodegenerative diseases. Selective chemical modification of the structure of NEPP11 may lessen its cytotoxicity observed at higher doses.

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Legends

Fig. 1. Chemical structure of NEPP11

Fig. 2. NEPP11 significantly blocks manganese-induced DNA fragmentation in PC12 cells. PC12 cells were preincubated with 5 μM NEPP11 for 30 min and treated with 0.25 mM $MnCl_2$ for another 20 h in D5 (DMEM + 5% FBS) medium. Soluble DNA was isolated and analyzed using agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. DNA was quantified with Intelligent Quantifier. Results are the mean of three or four experiments $\pm$ S.D. *$p < 0.05$ for difference from Mn. Similar results were obtained in the experiment using 0.5 mM $MnCl_2$ and 5 μM NEPP11.

Fig. 3. Effects of NEPP11 on manganese-induced signaling molecules. Western blot analysis of NEPP11 and/or manganese-treated PC12 cell extracts using antibodies against indicated proteins and their phosphorylated forms. PC12 cells were preincubated with 5 μM NEPP11 for 30 min and treated with 0.25 mM or 0.5 mM $MnCl_2$ for another 20 h in D5 medium. Whole-cell lysates (40 μg protein) were subjected to 10% SDS-PAGE and immunoblot as described in Materials and methods. Representative results are shown. Comparable results were achieved in several independent experiments.

Fig. 4 A. NEPP11 alone causes DNA fragmentation at high concentrations. PC12 cells were incubated with various concentrations of NEPP11 for 20 h in D5 medium. As a positive control, 0.25 mM $MnCl_2$ was used. Soluble DNA was isolated and analyzed using agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. B. NEPP11 is a strong activator of the JNK pathway at higher concentration. PC12 cells were incubated with various concentrations of NEPP11 for 20 h in D5 medium. As a positive control, 0.25 mM and 0.5 mM $MnCl_2$ were used. Western blot analysis was performed as described above. Representative results are displayed.

Fig. 5 A. SP600125 prevents manganese-induced DNA fragmentation in PC12 cells. PC12 cells were treated with SP600125 and $MnCl_2$ for 20 h in D4/7 (DMEM + 4% FBS + 7% HS) medium. Soluble DNA was isolated and analyzed using agarose gel electrophoresis and visualized under UV light after ethidium bromide staining. B. SP600125 inhibits c-Jun phosphorylation and increases ERK phosphorylation. PC12 cells were incubated for 20 h with various concentrations of SP600125 in the presence or absence of 0.5 mM $MnCl_2$. Western blot analysis was performed as described above.

Fig. 6. Model of signaling pathway for manganese-induced apoptosis