Neoechinulin A Impedes the Progression of Rotenone-Induced Cytotoxicity in PC12 Cells

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Neoechinulin A, an indole alkaloid from marine fungi, can protect PC12 cells from the cytotoxicity of 1-methyl-4-phenylpyridinium (MPP+), a Parkinson disease-inducing neurotoxin, by ameliorating downstream events resulting from mitochondrial complex I inactivation. However, the cytoprotective mechanisms remained unclear. In this study, by using rotenone, another parkinsonian-inducing neurotoxin targeting mitochondrial complex I, we investigated the cytoprotective mechanism of neoechinulin A. Rotenone-induced cell death was associated with accelerated glucose consumption, and excess glucose supplementation in the culture medium almost completely suppressed cell death, suggesting that glucose deficiency in the medium is critical for triggering cell death in this model. Co-treatment with neoechinulin A, but not neoechinulin A pre-treatment before rotenone exposure, significantly impeded cell death by rotenone. Although the presence of neoechinulin A did not affect the accelerated glycolytic turnover in rotenone-treated cells, it paradoxically decreased ATP levels in the cells, suggesting increased ATP consumption. Although the link between the decreased ATP levels and cytoprotection is not clear at present, it suggests that neoechinulin A may ameliorate rotenone toxicity by activating a cytoprotective machinery that requires ATP.

Key words neurodegeneration; mitochondria; reactive oxygen species; reactive nitrogen species; Parkinson disease; alkaloid

Parkinson disease (PD) is a progressive neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the substantia nigra of the brain.1) Currently, no cure is available that can impede the progress of neuronal cell death in PD. The majority of PD cases are sporadic with unknown etiology. However, since the identification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a by-product of illegal heroin synthesis, as a causative agent for an acute PD-like disorder among drug abusers and of its toxic metabolite 1-methyl-4-phenylpyridinium (MPP+) as an inhibitor of complex I (reduced nicotinamide adenine dinucleotide (NADH): ubiquinone oxidoreductase),2) mitochondrial toxins from the environment, including pesticides and herbicides, have gained attention as risk factors for sporadic PD. In fact, mitochondrial complex I dysfunction is associated with patients with sporadic PD.3,4)

Rotenone is used as a pesticide and as an inhibitor of mitochondrial electron transport chain complex I in various experimental studies. Similar to the case of MPP+ neurotoxicity, exposure to rotenone induces not only PD-like symptoms in experimental animals5) but also cytotoxicity along with pathological hallmarks including cytosolic protein aggregates in cultured neuronal cells.6,7) Nevertheless, the cytotoxic mechanism is not fully understood, and several mechanisms have been proposed, including energy failure, reactive oxygen production, and microtubule destabilization.8—12)

Neoechinulin A (Fig. 1) is an indole alkaloid isolated from marine fungi.13,14) Previously, we found that neoechinulin A can protect neuronal PC12 cells from cytotoxicity due to oxidant/nitrosative stress induced by the superoxide/nitric oxide co-generator 3-morpholinosydnonimine (SIN-1).15—15) Subsequently, we found that neoechinulin A can also protect PC12 cells from MPP+ by ameliorating downstream lethal events resulting from mitochondrial complex I inactivation.16) Although the cytoprotective mechanism of neoechinulin A remains unclear, it is probably not a single mechanism; while cytoprotection against SIN-1 can be afforded by pretreatment of cells with neoechinulin A at least for 12 h before SIN-1 challenge,13) protection against MPP+ toxicity does not require pretreatment but its co-presence during the neurotoxin treatment.16)

The present study was aimed at clarifying the cytoprotective mechanism of neoechinulin A in mitochondrial dysfunction-induced cell death. By using rotenone, we demonstrate here that neoechinulin A can also protect PC12 cells from rotenone cytotoxicity while paradoxically lowering cellular ATP levels. Possible links between the decreased ATP levels and the cytoprotection afforded by neoechinulin A against rotenone are discussed.

Fig. 1. Structures of Neoechinulin A and Preechinulin.
(A) Neoechinulin A, (B) preechinulin.

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MATERIALS AND METHODS

**Cells and Chemicals** Dulbecco’s modified Eagle’s medium (DMEM) (041-29775), RPMI 1640 (189-02025), NADH, glucose oxidase, and sodium pyruvate were purchased from Wako Chemicals (Osaka, Japan). Hydrazine, DL-lactate, NAD⁺, and 3,3’,5,5’-tetramethylbenzidine (TMB) were obtained from Nacalai Tesque (Kyoto, Japan). ATP, bovine serum albumin, Hanks’ balanced salt, and rotenone were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Lactate dehydrogenase (LDH) and horseradish peroxidase (HRP) were obtained from TOYOBO (Osaka, Japan). Other chemicals and salts were of analytical grade. Neocthinulin A and preechinulin (8,9-dihydronoechinulin A) were synthesized as described previously.14)

PC12 cells, a rat pheochromocytoma cell line, were routinely maintained in DMEM containing 5% horse serum, 10% fetal calf serum (FCS), glutamine (4 mM), penicillin G (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (FCS), glutamine (4 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml) for 4 days. The medium was changed every other day.

**Cell Treatment and Viability Assay** NGF-differentiated PC12 cells in 96-well plates were exposed to various concentrations of rotenone in RPMI medium containing NGF for various times, and viability was determined by LDH activity as described previously.17)

In brief, the medium was removed and live cells in the wells were lysed in 100 μl of lysis buffer (0.2% (v/v) Triton X-100, 0.2 mM diethylene pentaacetic acid (DETAPAC) in phosphate buffered saline (PBS)). The medium or the lysate was mixed with NADH and pyruvate in PBS at final concentrations of 200 μM and 0.5 mM, respectively, in a total volume of 200 μl in 96-well plates. NADH oxidation was monitored at 340 nm at 25 °C on a plate reader (Spectra Max, Molecular Devices). LDH leakage was expressed as % of LDH activity in medium per total activity of the corresponding culture well.

**Glucose Assay** Glucose concentration in the culture medium was measured in a 96-well assay plate by the stoichiometric conversion of glucose to hydrogen peroxide by glucose oxidase (GO), coupled with HRP-catalyzed oxidation of TMB.18) The final amount/concentrations of each constituent in 200 μl were as follows: 10 μl of cell culture medium, 0.5 mM TAM, 10 U/ml of HRP, 2 U/ml of GO, and 0.1% (v/v) Triton X-100 in PBS. The reaction was carried out under aerobic condition at 37 °C for 30 min. Absorbance at 450 nm was recorded in a plate reader (Spectra Max). H-Glucose diluted in PBS was used as a standard. The glucose concentration of the basal cell culture medium used in this study (RPMI 1640) was 11 mM, and after addition of FBS to 10%, it was approximately 10 mM.

**Lactate Assay** Lactate levels in the culture medium were assayed in a 96-well assay plate by the production of NADH catalyzed by LDH.19) In brief, 100 μl of 20-fold diluted culture medium in PBS was mixed with an equal amount of reaction mixture containing 0.5 M glycine, 0.4 M hydrazine, 1 mM DETAPAC, 4 mM NAD⁺, and 0.5% Triton X-100, without or with 20 U/ml of LDH. The reaction was carried out at 37 °C for 1 h, and absorbance at 340 nm was measured in a plate reader (Spectra Max). LDH-dependent increases in the absorbance were converted to l-lactate concentration by using an external standard curve made with DL-lactate. l-isomer content was assumed as 50%.

**ATP Assay** Cellular ATP content was determined using a luciferin-luciferase assay kit (Toyo B-Net, Tokyo, Japan). After treatment, cell culture medium was removed and assay reagent was immediately added together with an equal amount of PBS. Luminescence was measured in a luminometer (LB96Y, PerkinElmer, U.S.A.).

**Statistics** Data are expressed as means±S.D. Student’s t-test or one-way analysis of variance (ANOVA) followed by the Tukey’s test was used as appropriate for statistical analysis.

RESULTS AND DISCUSSION

**Glucose Shortage, in Conjunction with Complex I Inhibition, Is a Crucial Factor for Cell Death Induced by Rotenone** In rotenone cytotoxicity, incubation periods with the toxicant required for cell death vary considerably in the literature. Therefore, we first addressed the mechanistic aspects of cell death induced by rotenone under our assay conditions. When NGF-differentiated PC12 cells in 96-well plates were exposed to increasing concentrations of rotenone in 50 μl of medium per well for 25 h, more than 80% of the cells were killed at concentrations of 0.4 to 10 μM (Fig. 2A). However, when identical numbers of cells were treated with the same concentrations of rotenone in 100 μl of medium per well, cytotoxicity was dramatically diminished throughout the concentration range. However, when the treatment was continued further without changing culture medium, the rotenone-treated cells in 100 μl of medium were also almost completely killed within the next 24 h (data not shown). These results suggest that the amount of culture medium per cell inversely correlated with the incubation period required for the onset of rotenone-induced cell death, which could be a factor behind the inconsistency of incubation time for rotenone cytotoxicity in the literature.

To clarify the dependency of rotenone cytotoxicity on glucose concentration in a 96-well assay plate by the stoichiometric conversion of glucose to hydrogen peroxide by glucose oxidase (GO), coupled with HRP-catalyzed oxidation of TMB.18) The final amount/concentrations of each constituent in 200 μl were as follows: 10 μl of cell culture medium, 0.5 mM TAM, 10 U/ml of HRP, 2 U/ml of GO, and 0.1% (v/v) Triton X-100 in PBS. The reaction was carried out under aerobic condition at 37 °C for 30 min. Absorbance at 450 nm was recorded in a plate reader (Spectra Max). H-Glucose diluted in PBS was used as a standard. The glucose concentration of the basal cell culture medium used in this study (RPMI 1640) was 11 mM, and after addition of FBS to 10%, it was approximately 10 mM.
These results indicate that, compared with control, rotenone treatment increased cellular glucose consumption approximately by 25%. It should be noted that although the amount of glucose in the medium was depleted in control cells without glucose supplementation, no cytotoxicity could be observed. Taken together, these results suggest that glucose availability per cell is a factor responsible for the dependency of rotenone cytotoxicity on the amount of culture medium. However, glucose depletion alone is not sufficient for cell death. Rather, impairment of mitochondrial functions in conjunction with glucose shortage is essential for the manifestation of rotenone-induced cytotoxicity in PC12 cells under our assay conditions. This finding is consistent with some previous reports.\textsuperscript{20,21)}

**Neoehcinulin A Can Delay the Onset of Rotenone-Induced Cell Death**

Having gained insight into the mechanistic aspects of rotenone-induced cell death under our assay conditions, the effects of neoehcinulin A on the cytotoxicity were measured. In this experiment, we also assessed the effect of a neoehcinulin A analog that lacks cytoprotective activity against SIN-1 (superoxide/nitric oxide co-generator) due to conversion of the C8/C9 double bond into a single bond (preechcinulin, Fig. 1).\textsuperscript{14) As shown in Fig. 3A, the presence of neoehcinulin A (100 µM) during rotenone exposure dramatically diminished cytotoxicity; at 21 h after treatment while LDH leakage exceeded 70% in rotenone treated cells, it remained below 25% in cells co-treated with neoehcinulin A. However, the cells incubated with neoehcinulin A were not completely resistant to rotenone; in the next 10 h, rotenone plus neoehcinulin A co-treated cells also died (data not shown). In contrast to neoehcinulin A, preechcinulin showed no cytoprotection. The results demonstrate that in addition to MPP\textsuperscript{+}-induced cytotoxicity,\textsuperscript{16) neoehcinulin A is also effective against rotenone-induced cytotoxicity and that the presence of the C8/C9 double bond, which gives rise to a conjugated system across the indole moiety to the diketo-
piperazine ring, is essential for the cytoprotective action of neoechinulin A, as observed for SIN-1-induced cytotoxicity.\textsuperscript{14) Regarding SIN-1-induced cytotoxicity, pretreatment with neoechinulin A for at least 12 h is essential for cytoprotection.\textsuperscript{13) Therefore, we next assessed whether neoechinulin A pretreatment alone could also afford cytoprotection against subsequent rotenone treatment. As shown in Fig. 3B, neoechinulin A pretreatment for 24 h alone provided marginal protection against rotenone-induced cytotoxicity, whereas successive addition of neoechinulin A upon rotenone exposure showed a protective effect to a similar extent as co-treatment alone. Thus, the requirement of the simultaneous presence of the alkaloid with the neurotoxin for cytoprotection is consistent with the case of MPP\textsuperscript{+}-induced cytotoxicity.\textsuperscript{16) These results suggest that antioxidant activity\textsuperscript{14) and/or interaction of an unknown cellular target molecule with neoechinulin A plays a role in the cytoprotective action of neoechinulin A against rotenone.\textsuperscript{\textemdash}Neoechinulin A Decreases Steady-State Cellular ATP Levels without Affecting Rotenone-Mediated Increases in Glycolytic Turnover Under our assay conditions, rotenone cytotoxicity was critically dependent on glucose availability to cells (Fig. 2). To clarify the cytoprotective mechanism of neoechinulin A, glucose consumption, lactate production, and cellular ATP levels were measured during a period before the onset of cell death (asymptomatic phase: up to 12 h, Fig. 3A). Rotenone treatment increased not only glucose consumption (Fig. 4A), but also lactate production compared to control cells, demonstrating that the glycolytic pathway compensated for the compromised oxidative phosphorylation for ATP synthesis in rotenone-treated cells. However, the presence of neoechinulin A did not affect glucose consumption and lactate production in rotenone-treated cells, indicating that neoechinulin A did not affect the glycolytic turnover that had been stimulated by rotenone.

Cellular ATP levels in the asymptomatic phase were also measured at 12 h after treatment (Fig. 4C). Compared with control cells, ATP levels in rotenone-treated cells were slightly (approximately 10%) decreased. However, ATP levels in cells co-treated with rotenone and neoechinulin A were decreased to 50% of the control levels, a level much lower than that induced by rotenone treatment alone. Similar results were observed as early as 4 h after treatment (data not shown). Because cellular ATP levels in general are in steady state, which is determined by the balance between the rates of ATP synthesis and degradation, the decrease in ATP levels in rotenone-treated cells is likely attributable to compromised oxidative phosphorylation for ATP synthesis.
of production and consumption, the decreased ATP levels suggest that neoechinulin A could have either increased ATP consumption and/or decreased ATP synthesis. Because mitochondria can no longer generate substantial amounts of ATP by using NADH as a substrate in the presence of this concentration of rotenone, as assessed by almost complete suppression of cellular O₂ consumption as measured with an O₂ electrode (data not shown), ATP synthesis could largely be dependent on the glycolytic pathway. Because the rotenone-induced elevation in glycolytic turnover did not change in the presence of neoechinulin A (see Fig. 4), the decline in steady-state ATP levels in cells co-treated with rotenone and neoechinulin A could largely be attributed to increased ATP consumption by the cells.

A couple of possibilities may account for the paradoxically decreased ATP levels. When electron flow in the mitochondrial electron transport chain is impaired, ATP synthase in mitochondria (complex V) hydrolyzes ATP and pumps protons from the matrix to the intermembrane space to prevent the collapse of mitochondrial inner membrane potential, thereby preventing cell death.2,11 To assess whether the decreased ATP levels induced by neoechinulin A in rotenone-exposed cells may have resulted from ATP hydrolysis by complex V, the effect of oligomycin was measured. If ATP hydrolysis occurs at complex V, the addition of oligomycin could reverse the decline in ATP levels.23 Oligomycin treatment, however, slightly decreased the ATP levels further in neoechinulin A plus rotenone-treated cells (data not shown), suggesting that mitochondrial ATP hydrolysis may not be responsible for the decreased ATP levels, and that mitochondria still generate a small portion of ATP by using electrons from entities other than complex I. Another potential demand for ATP in relation with cytoprotection against rotenone is for the process of removal and repair of damaged cellular components. In this regard, rotenone treatment has been demonstrated to cause accumulation of cytosolic proteins,6,7,23,24 Generally, heat shock proteins25 and the ubiquitin-dependent proteasomal/autophagy system26 contribute to disaggregation and degradation of protein aggregates, respectively. Neoechinulin A may activate these ATP-dependent cytoprotective machineries, resulting in the decrease in ATP content. Studies are underway to address these possibilities.

Neoechinulin A Does Not Preserve ATP During the Progression of Cell Death Evidence suggests that a certain level or even an elevated level of ATP is required for the execution of apoptotic cell death.2,23 Conversely, a decline in ATP levels below a certain critical threshold level triggers necrotic cell death.28,29 Previous studies demonstrated that necrosis in neuronal cells caused by mitochondrial toxins including rotenone, as indexed by LDH leakage, is closely associated with severe ATP depletion.9,20,30,31 We next measured the ATP levels in a later time period, wherein rotenone alone causes severe cell death and the co-treatment with neoechinulin A mitigates its toxicity. After a 24-h treatment, rotenone induced substantial cytotoxicity, and the ATP levels in the rotenone-treated cells decreased to approximately 10% of the control levels (Fig. 5). Despite significant cytoprotection, the ATP levels in neoechinulin A and rotenone co-treated cells were also severely decreased to the same levels as those of rotenone-treated cells. Thus, neoechinulin A does not preserve ATP levels while impeding the progression of cell death. It is conceivable therefore that decreased cellular damage afforded by ATP-dependent cytoprotective machinery during the asymptomatic period, as discussed above, may contribute to cell survival under this minimum level of ATP. To our knowledge, this paradoxical cytoprotection with neoechinulin A regarding ATP levels is unprecedented among cytoprotective natural compounds reported so far. Studies are underway to clarify how neoechinulin A facilitates ATP consumption in the initial phase without preserving ATP in the late phase while prolonging cell survival.

In conclusion, the present study demonstrates that rotenone-induced PC12 cell death is significantly affected by glucose availability. Neoechinulin A can significantly delay the onset of rotenone-induced PC12 cell death when present simultaneously with the toxin. The presence of the C8/C9 double bond in neoechinulin A is an essential structural requirement for cytoprotection against rotenone. Neoechinulin A does not affect the glycolytic turnover in the rotenone-treated cells but decreases the ATP levels, suggesting increased ATP consumption in these cells. Taken together, these results suggest that neoechinulin A may activate cytoprotective machinery that requires ATP, thereby delaying the progression of cytotoxicity.

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