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Neoechinulin A Protects PC12 Cells against MPP^+-induced Cytotoxicity

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Abstract Neoechinulin A, an alkaloid from *Eurotium rubrum*, can protect neuronal PC12 cells against cytotoxicity of a potent oxidant, peroxynitrite. Because involvement of peroxynitrite has been suggested in the pathogenesis of Parkinson's disease, we assessed whether this alkaloid could also protect PC12 cells from the cytocidal action of 1-methyl-4-phenylpyridine (MPP^+), a neurotoxin capable of provoking acute Parkinson's-like neurodegeneration in humans. Neoechinulin A could protect PC12 cells from MPP^+ cytotoxicity without protecting against mitochondrial complex I dysfunction, suggesting the alkaloid can ameliorate downstream events of mitochondrial failure. Thus, neoechinulin A has the potential to intervene in this progressive neurodegeneration.

Keywords alkaloid, reactive oxygen/nitrogen species, neurodegeneration, Parkinson's disease

We previously reported that neoechinulin A (Fig. 1), an indole alkaloid from the fungus *Eurotium rubrum* Hiji025, can protect neuronal PC12 cells from death induced by peroxynitrite ($ONOO^-$) [1, 2]. Although neoechinulin A has potent scavenging activity against $ONOO^-$, the neuroprotection conferred by this alkaloid depends on unknown biological effects rather than antioxidant activity [2].

Parkinson's disease, which affects 0.1% of the population over the age of 40 years, is characterized by a progressive loss of dopaminergic neurons in the substantia nigra [3, 4]. Currently, no cure is available that can impede neuronal cell death. Except for some inherited cases, causative factors for the vast majority of cases remain elusive. The environmental toxin hypothesis has gained attention since the identification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of illegal heroin synthesis, as a causative agent for an acute Parkinson's-like disorder among drug abusers [5]. In the brain, MPTP is oxidized to 1-methyl-4-phenylpyridine (MPP^+; Fig. 1), which is then taken up by neurons of the substantia nigra *via* the dopamine transporter [3]. In cells, MPP^+ accumulates in mitochondria and binds to complex I of the electron transport chain, resulting in the production of reactive oxygen species (ROS) by the organelle, a decline in ATP biosynthesis, and eventually the demise of the cells [6, 7]. A few insecticides and herbicides that also target mitochondrial complex I have been demonstrated to induce Parkinsonism in animal models, supporting the role of environmental toxins in the neurodegeneration [4]. MPTP and MPP^+ have been widely used in studies of Parkinson's disease in rodents and in cultured cells, respectively.

As peroxynitrite has been suggested to be involved in the pathogenesis of Parkinson's disease as well as MPP^+ cytotoxicity in animals and cultured cells [3, 8, 9], we

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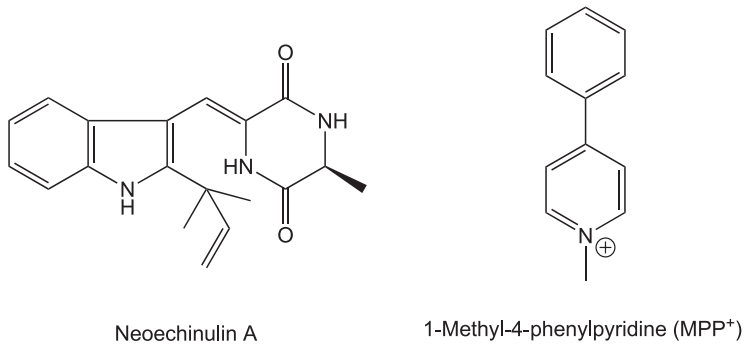

Fig. 1 Chemical structures of neoechinulin A and MPP^+.

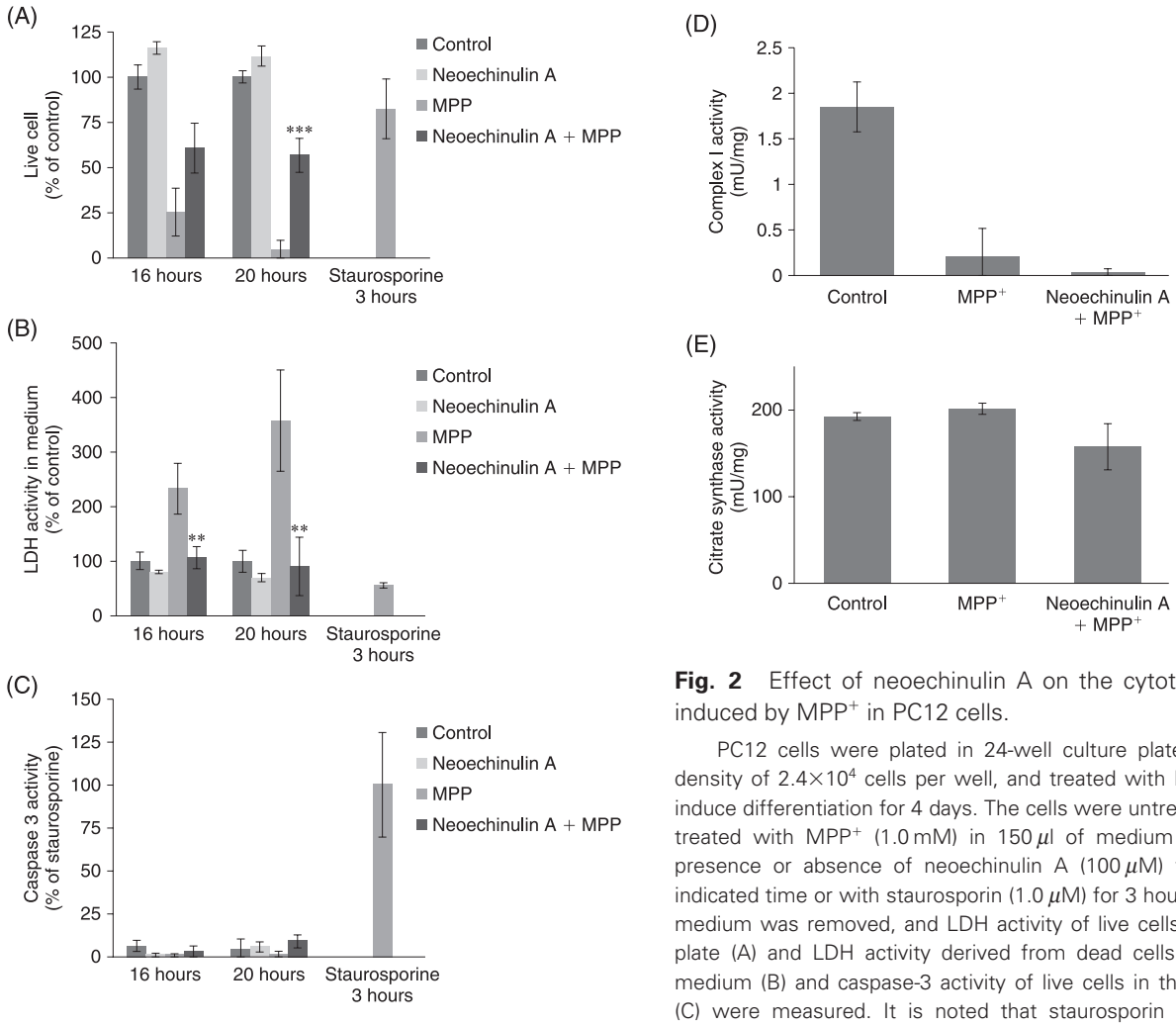

Fig. 2 Effect of neoechinulin A on the cytotoxicity induced by MPP^+ in PC12 cells.

PC12 cells were plated in 24-well culture plates at a density of 2.4×10^4 cells per well, and treated with NGF to induce differentiation for 4 days. The cells were untreated or treated with MPP^+ (1.0 mM) in 150 μl of medium in the presence or absence of neoechinulin A (100 μM) for the indicated time or with staurosporin (1.0 μM) for 3 hours. The medium was removed, and LDH activity of live cells in the plate (A) and LDH activity derived from dead cells in the medium (B) and caspase-3 activity of live cells in the plate (C) were measured. It is noted that staurosporin caused sudden cell detachment after 3 hours. Values are means±intra-assay deviations expressed as SD from 3 well cultures in a representative experiment. Values for MPP^+ plus neoechinulin A-treated cells are significantly different compared to those of MPP^+-treated cells (***, $P<0.001$; **, $P<0.01$).

For the assessment of mitochondrial functions (D and E), PC12 cells in 100 mm culture dishes (2.5×10^5 cells/dish) were differentiated with NGF as above. The cells were treated with 5.0 ml of MPP^+ (1.0 mM) in medium in the absence or presence of neoechinulin A (100 μM) for 12 hours. Cells were washed with PBS, and lysates were prepared using 0.2% laurylmaltoside. Complex I activity (D) was measured as the rate of rotenone-sensitive NADH oxidation with decylubiquinone [12]. Citrate synthase activity (E) was measured by means of the 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) reduction-coupled detection of coenzyme A generation from the reaction of acetyl coenzyme A and oxaloacetate [13]. Values are means±intra-assay deviations expressed as SD from 3 cultures in a representative experiment.

assessed whether neoechinulin A could also protect PC12 cells against neuronal cell death induced by MPP^+. Exposure of nerve growth factor (NGF)-differentiated PC12 cells to MPP^+ resulted in cell death that began approximately 12 hours after exposure and continued to increase over the next 8 hours. Simultaneous addition of neoechinulin A (100 μM) with MPP^+ dramatically attenuated MPP^+ cytotoxicity. At 20 hours, where MPP^+ treatment alone had almost completely killed cells, nearly 60% of cells survived when neoechinulin A was also present (Fig. 2A). The cell death caused by the neurotoxin mostly resulted from necrosis as a significant release of lactate dehydrogenase (LDH) into the medium could be observed while no caspase-3 activation in the cytoplasm of the dying cells was found. This contrasted with the response to staurosporin, a well-established inducer of apoptosis (Fig. 2B and C). No change in the absorption spectrum was observed when neoechinulin A was incubated with MPP^+ in a physiological solution (PBS, pH 7.4) at 37°C overnight (data not shown), ruling out the possibility that neoechinulin A reacted with MPP^+ and thereby chemically antagonized the neurotoxin.

Previous studies indicated that pre-incubation with neoechinulin A for 12 hours was essential to render the cells resistant to peroxynitrite toxicity, and that once the cells had been fortified by neoechinulin A treatment, the presence of the alkaloid during exposure to the oxidant was not essential for cytoprotection [1, 2]. Interestingly however, when cells were pre-incubated with neoechinulin A for 24 hours, and then exposed to MPP^+ in fresh medium (without neoechinulin A), no cytoprotection was observed (data not shown). Instead, simultaneous addition of neoechinulin A upon MPP^+ exposure was sufficient for protection against MPP^+ cytotoxicity, as shown in Fig. 1.

Irreversible inhibition of mitochondrial NADH-ubiquinone oxidoreductase (complex I) resulting from ROS production has been suggested as an initial event in MPP^+-induced neuronal cell death [10, 11]. We therefore examined whether neoechinulin A could mitigate complex I dysfunction. MPP^+ treatment for the sub-lethal exposure time of 12 hours almost completely abolished complex I activity (Fig. 2D). In contrast, the activity of citrate synthase was unaffected in MPP^+-treated cells (Fig. 2E). Co-incubation of neoechinulin A with MPP^+ however, did not prevent the decline in complex I activity (Fig. 2A). Therefore, the mechanism of cytoprotection by neoechinulin A apparently results from preventing cytotoxic events that are either downstream or independent of complex I dysfunction.

We have found that neoechinulin A treatment can enhance the capacity of cells to produce NADH [1, 2], which may be one factor underlying the cytoprotective mechanism. The requirement for the co-presence of neoechinulin A during MPP^+ treatment to provide protection suggests that the antioxidant activity of neoechinulin A [1, 2] and/or interaction with a cellular target may be responsible for the cytoprotection against MPP^+. Studies of the underlying mechanisms are in progress. However, given that complex I dysfunction is a common feature of sporadic Parkinson's disease [3], the present study suggests that neoechinulin A or its derivatives has potential as a therapeutic intervention for this progressive neurodegenerative disorder.

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