論　文　内　容　要　旨

Nicotine-induced upregulation of miR-132-5p enhances cell survival in PC12 cells by targeting the anti-apoptotic protein Bcl-2

（ニコチンにより誘導される miR-132-5p は抗アポトーシス分子 Bcl-2 を標的とすることにより PC12 細胞の生存率を向上させる）

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Background and purpose

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by gradual and irreversible cognitive deterioration where apoptosis plays a critical role. Currently available drugs are aimed at enhancing cholinergic transmission providing only symptomatic treatment. Studies have indicated that nicotinic acetylcholine receptor (nAChR) agonists such as nicotine have neuroprotective effects, however, the exact mechanism is not yet elucidated.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene-expression in sequence-specific manner. In the CNS, miRNAs are involved in neurogenesis, neuronal differentiation, and apoptosis. Studies have demonstrated that nicotine exposure modulates the expression pattern of miRNAs.

The aim of our study was to identify miRNAs specifically regulated by nicotine and elucidate the exact cellular pathways of neuroprotection.

Methods

PC12 cells were treated with nicotine (100 μmol/l) or NGF (50 ng/ml) for 2 and 9 days, respectively and a miRNA microarray analysis was done using 3D-Gene Extraction software (TORAY). MiRNAs with at least 1.5-fold change in expression were selected. For experimental validation with RT-qPCR of miRNA, cells were treated with nicotine for 4 h and with NGF for 48 h.

For assessment of cell viability, PC12 cells were seeded in normal serum-containing medium; then, the medium was replaced with serum-free medium containing nicotine. For transfection, PC12 cells were seeded in normal serum-containing medium; then, transfected with miR-132-5p mimic or its inhibitor followed by serum starvation for 48 h. Cell viability was measured by cell counting kit-8. Absorbance was measured at 450nm by a microplate reader.

The protein expression of CREB, pCREB, Bcl-2, Bax, cleaved caspse-3 and α-tubulin was determined by western-blotting analysis. The band intensity was quantified using Image-J software.

Results

The miRNA-microarray results showed 12 miRNAs upregulated in both nicotine and NGF-treated groups. Among these, miR-132-5p, a brain-enriched miRNA shown to be

downregulated in degenerative conditions, was increased by 1.67 fold in the nicotine-treated and by 2.79 fold in the NGF-treated group. Further validation with RT-qPCR showed a 1.5-fold increase in the miR-132-5p level upon nicotine treatment.

To test whether the nicotine-induced increase in the miR-132-5p level occurs through CREB signaling, PC12 cells were treated with nicotine for 0–240 h. The treated cells showed >2 fold increase in CREB-phosphorylation, peaking at 4 h.

To explore the effect of nicotine on cell viability, cells were treated with or without nicotine under serum starvation for 48 h. CCK-8 assay revealed an increase in cell viability from 35% to 54% in nicotine-treated group.

To test whether the protective effects of nicotine are associated with regulation of anti-apoptotic protein Bcl-2, cells were treated with or without nicotine for 48 h. Nicotine treatment significantly increased Bcl-2 immunoreactivity by 1.4 fold. In contrast, Bax immunoreactivity was decreased by 1.3 fold suggesting the regulation of apoptotic pathway. To further elucidate the effect of nicotine on cell viability, cleaved –caspase activity was detected using western blot analysis. Nicotine treatment significantly decreased the cleaved caspase-3 immunoreactivity by 1.7 fold.

To evaluate the effect of miR-132-5p on cell survival, cells were transfected with miR-132-5p mimic or its inhibitor. miR-132-5p overexpression increased cell viability from 38% to 70% while the inhibitor exhibited a decrease in cell viability from 38% to 25%.

To determine whether the protective effect of miR-132-5p is mediated by Bcl-2 and Bax, PC12 cells were transfected with miR-132-5p mimic or its inhibitor.. Western blot analysis showed 3.9-fold increase in Bcl-2 after 48 h of transfection with miR-132-5p mimic and no change after transfection with its inhibitor. No change in Bax immunoreactivity was observed. To further elucidate the effect of miR-132-5p on cell viability, cleaved caspase-3 activity was assessed. Western blot analysis showed 1.25-fold decrease in the expression level of cleaved caspase-3 after 48 h of transfection with the miR-132-5p mimic and a 2.5–fold increase after transfection with its inhibitor.

Discussion

In this study, we identified miR-132-5p selectively regulated by nicotine and NGF. MiR-132-5p plays an important role in neurodevelopment and is linked to various neurodegenerative disorders. The entire miR-132/-212 cluster has been found to be altered in the cerebral cortex of late-onset AD.

Studies have demonstrated the CREB pathway as a final-common intracellular signaling pathway. Our results showed an increase in CREB-phosphorylation after nicotine treatment, and the expression of miR-132-5p was significantly ($p = 0.003$) upregulated at the time-point of maximum CREB phosphorylation (i.e., 4 h) suggesting that nicotine-induced upregulation of miR-132-5p is mediated by pCREB.

Nicotine treatment for 48h had a protective effect on PC12 cells when placed under serum starvation conditions. Furthermore, miR-132-5p overexpression lead to a significant increase, whereas miR-132-5p inhibitor resulted in a marked decrease in cell viabilityshowing that nicotine-induced neuroprotection is mediated by upregulation of miR-132-5p.

Our data showed an increase in Bcl-2 expression upon nicotine treatment. Overexpression of miR-132-5p resulted in 3.9-fold increase in Bcl-2 expression. Also, miR-132-5p mimic decreased cleaved caspase-3 expression, while its inhibitor increased its expression. Regulation of cleaved caspase-3 by this miRNA explains the decrease in cell viability from 38% to 25%. These results show that nicotine-induced neuroprotection is mediated by upregulation of miR-132-5p, which in turn results in overexpression of Bcl-2. Our study showed a decrease in the expression of Bax, following treatment with nicotine with subsequent increased cell survival. However, treatment with miR-132-5p mimic or inhibitor resulted in no change in Bax expression level. These results suggest that the nicotine-induced increased cell survival is also mediated by Bax regulation; independent of miR-132-5p and is regulated via alternative pathway.
Studies have shown the potential therapeutic use of neurotrophins in the repair and regeneration of nervous system; however, direct use is limited because of their low permeability through the blood-brain barrier (BBB). In such a scenario, nAChR agonist has promising potential as a neurotherapeutic agent as it readily crosses the BBB, upregulates miR-132-5p and elicits neuroprotection by regulation of apoptotic proteins.

In conclusion, our results showed that nAChR-induced neuroprotection is mediated by increased expression of miR-132-5p, which regulates Bcl-2 as a downstream target for cell survival in PC12 cells.