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뇌신경세포사멸과 관련된 3-Hydroxykynurenine (3-HK)의 작용에 대한 NF-κB의 보호효과

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NF-κB attenuates 3-Hydroxykynurenine (3-HK)-induced apoptosis.

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Objectives

3-Hydroxykynurenine (3-HK), kynurenine pathway metabolite, is known as a endogenous neurotoxin, which maybe associated with several neurodegenerative disorder. To investigate the molecular mechanism of neurodegeneration induced by 3-HK, we examined the effect of 3-HK on SK-N-SH cells. Furthermore, we investicated roles of NF-κB in 3-HK-induced cell death.

Materials and Methods

Cell culture SK-N-SH, human neuroblastoma cells, were cultivated at 37℃ in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) in a humidified 95% air, 5% CO_2 incubator. Cells were cultured in RPMI 1640 containing 1% FBS for 2 h before 3-hydroxykynurenine (250 mM) treatment to assure neuronal survival and the morphological integrity of cells.

Cell Viability Assay (alamarBlue test) SK-N-SH cells were plated on 96-well plates (Nunc, Denmark) at a density of 15,000 cells/well, in 100 ml of 10% FBS/RPMI 1640 and incubated for 24 h. Before 2 h 3-HK treatment, the media was replaced with 1% FBS/RPMI 1640. At the end of the treatment, 10 uml of alamarBlue (Serotec, UK) was aseptically added. The cells were incubated for 3 h and absorbance of the cells was measured at a wavelength of 570 nm with an ELISA Reader (Molecular Devices, Sunnyvale, CA). The background absorbance was measured at 600 nm and was subtracted. The cell viability was defined as [(test sample count)-(blank count)/(untreated control count)-(blank count)]100.

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Hoechst 33258 staining The SK-N-SH cells were fixed with 4% paraformaldehyde for 20 min and stained with 8 μg/ml of Hoechst dye 33258 for 5 min. They were washed twice with PBS and analyzed under fluorescent microscopy. The dead cells were characterized by their fragmented nuclei, and the apoptotic morphologic changes were characterized by chromatin condensation and the formation of apoptotic bodies.

Determination of ROS generation Cultured cells were treated with 10 μM of DCF-DA (6-carboxy-2',7'-dichloro-dihydrofluorescein diacetate, dicarboxym-ethylester) dissolved in HCSS buffer (20 mM HEPES, 2.3 mM CaCl2, 120 mM NaCl, 10 mM NaOH, 5 mM KCl, 1.6 mM MgCl2, 15mM glucose) and 2% Pluronic F-127 at 37℃ for 30min. DCF fluorescence generated by free radicals in cells were estimated using Olympus IX70 microscope with mercury lamp flouresence (Exc. 485 nm, Emi. 530 nm), captured by CCD camera. Then, image analysis was performed using NIH Image 1.65 program and was measured using Flow cytometry (TECAN, GENios, Maennedort, Switzerland) with excitation at 485 nm and emission at 530 nm.

Immunoblotting Cells, collected using cytospin, were fixed with 4% paraformaldehyde in 0.1 μM phosphate-buffered saline (pH 7.4) for 10 min. They were treated with 0.3% H_2O_2 in 100% EtOH for 10 min to eliminate endogenous peroxidase and incubated with 10% normal goat serum (ABC kit, Zymed, South San Francisco, CA, USA) for 1 hr at 37°C. Cells were incubated with first antibodies (1:100) in 10% normal goat serum 1 hr at 37°C. After primary incubation, cells were treated with biotinylated secondary antibodies (ABC kit) for 1 hr at 37°C, followed by incubation with a streptavidin peroxidase conjugate (ABC kit) for 1 hr at 37°C. Immunolabels were visualized with 3,3-diaminobenzidine staining kit (Zymed) and counter-stained with Meyer's Hematoxylin.

Caspase activity assay In order to assay caspase activity in SK-N-SH cells, 10,106 cells were harvested from each P100 plate and lysed with 1ml lysis buffer (10 mM Tris-Hcl, pH 7.4, 10 mM NaH_2PO4, pH 7.4, 130 mM NaCl, 1% Triton X-100, 10 mM NaF). 50 μl of lysate was added into 200 μl of HEPES buffer(40 mM HEPES, pH 7.5, 20% glycerol, 4 mM DTT) with 0.25 mM aVAD-PNA, pan caspase substrate for 1 hr. Caspase activity was measured using ELISA Reader(Molecular Devices) with absorbency at 405nm.

Results and Discussion

The results may be summarized as follows: 1. 3-HK induces neuronal cell death on dose and time dependent manners in SK-N-SH cells, 2. NF-κB activation is involved in 3-HK induced signal transduction, Consequently, NF-κB activation palys a protective role in 3-HK-induced apoptosis and acts and upstream regulators of caspase.