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뇌신경세포사멸을 유도하는 수산화도파민의 작용에 대한 키누레닌산의 보호효과

농촌과학기술원 : 이광길, 여주홍
서울대학교 보라매병원 : 이상형
중앙대학교 의과대학 : 김성수
(주) 바이오그랜드 : 윤유식

6-hydroxydopamine-induced neuronal cell death and its protection by Kynurenic acid (KYNA)

[1]Applied Sericulture & Apiculture Division, National Institute of Agricultural Science and Technology [2]Department of Neurosurgery, College of Medicine, Neuroscience Research Institute, Seoul National University [3]Department of Anatomy and Cell biology, College of Medicine, Chung-Ang University [4]Biogrand Inc.,
Kwang Gill Lee[1], Joo Hong Yeo[1], Sang Hyung Lee[2], Sung Su Kim[3], Yoo Sik Yoon[4,*]

Objectives

Kynurenic acid(KYNA), a tryptophan metabolite, is known to have cell protective effect against various insults. But so far, the protective mechanism is largely unknown. In this study, we investigated how the KYNA exerts protective effect against 6-OHDA, acausative molecule of Parkinson syndrome, using SH-SY5Y cells. Incubation of SH-SY5Y cells with 6-OHDA induced reactive oxygen species(ROS), increased[Ca2+]i and evoked apoptosis. Moreover scavenging of ROS with antioxidant NAC attenuated [Ca2+]i increase, while chelating [Ca2+]i with BAPTA-AM did not. This result implicates that ROS regulates [Ca2+]i levels in the cell death.

Interestingly ,pretreatment of KYNA reduced significantly the ROS and [Ca2+]i , eventually inhibited the cell death. Our result suggested the ROS and [Ca2+]i mediated 6-OHDA induced cell death and KYNA down-regulated ROS and [Ca2+]i , leading to inhibiting the cell death.

Materials and Methods

Cell culture SK-N-SH cells, the human neuroblastoma cells, were plated on PEI-coated 96-well plates at a density of 40,000 cells/well in the media of 10% FBS/DMEM to perform MTT reduction assay. At 2 hr before experiment, the media was replaced with the low serum media(DMEM with 1% FBS), and each material was treated for appropriate time.

주저자 연락처(Corresponding author):윤유식 E-mail:thanks@cau.ac.kr Tel :02-813-5387

Determination of ROS generation Cultured cells were treated with 10 uM 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. 488 nm, Emi. 510 nm), captured by CCD camera. Then, image analysis was performed using NIH Image 1.65 program and was measured using Flow cytometry (GENios, Tecan, NC, USA) with excitation at 485 nm and emission at 510 nm.

Hoechst 33258 staining DNA-bindingfluorochrome bis-benze (Hoechst 33258 dye) was used to observe morphological changes of nuclear chromatin in apoptotic cells. 0.5-3.0 * 106 cells were centrifused for 10 min and collected. After being washed with PBS, these cells were fixed for 10 min and followed by fixation with 50 μg/ml paraformaldehyde. Samples were washed with PBS, stained with 16 g/ml of Hoechst dye 33258 for 15 min, washed again with distilled water. Then, 10 ul aliquots were plated on a slide glass to visualize changes of apoptotic chromatin under a fluorescent microscope.

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 NaH2PO4, 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.

Analysis of mitohondrial membrane potential ($\Delta\Psi_m$) The changes in mitochondrial membrane potential ($\Delta\psi_m$) were estimated using tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Eugene, OR, USA) , which is a cationic potentipmetric dye that accumulates preferentially into energized mitochondria driven by the membrane potential. To estimated of $\Delta\psi_m$, cells were incubated with 100 nM TMRE for 15 min at 37℃ and then TMRE fluroscein intensity was measured with excitation at 549 nm and emission at 574 nm using a flurorometer (TECAN. GENios ,Maennedort, Switzeland). Intensity of $\Delta\psi_m$ is expressed as arbitary untis of ralative value. Fluroscence image was observed using an IX70 microscope (Olympus. Tokyo, Japan) equipped with attachments for fluroscnce microscopy.

Results and Discussion

6-OHDA evokes massive oxidative stress, which induces elevation of cytosolic Ca2+ concentration. Also, pretreated KYNA inhibits increase of cytosolic Ca2+ conc.

6-OHDA collapses mitochondrial membrane potential.

KYNA, antagonist of NMDA receptor, protects mitochondrial malfunction through blocking generation of ROS induced by 6-OHDA.