Neuroscience Letters 633 (2016) 94–100

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Insulin on activation of autophagy with integrins and syndecans against MPP+-induced α-synuclein neurotoxicity

Mahesh Ramalingam, Sung-Jin Kim (Ph.D.) (Prof.)*

Department of Pharmacology and Toxicology, Metabolic Diseases Research Laboratory, School of Dentistry, Kyung Hee University, Seoul 02447, Republic of Korea

HIGHLIGHTS

- Insulin inhibited MPP+-induced cell membrane damages.
- Insulin also inhibited the Cox-2 and α-synuclein levels.
- MPP+ and/or insulin enhanced the autophagy LC3.
- Insulin enhanced the ILK and induced the integrins β3, αV, syndecan-1 and -3.
- Insulin prevents MPP+-induced apoptosis in SH-SY5Y + RA cells.

ARTICLE INFO

Article history:
Received 18 March 2016
Received in revised form 18 July 2016
Accepted 15 September 2016
Available online 16 September 2016

Keywords:
Parkinson's disease
Neurodegenerative
SH-SY5Y
Cox-2
ILK

ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease in the elderly caused by dopaminergic neuronal cell death. Human neuroblastoma SH-SY5Y cells differentiated by retinoic acid have been used to study the *in vitro* PD model induced by 1-methyl-4-phenyl pyridinium (MPP+). In this study, pretreatment of insulin inhibited MPP+-induced cell membrane damages, which also inhibited the Cox-2 and α-synuclein levels. In addition, MPP+ and/or insulin enhanced the autophagy LC3. Furthermore, MPP+-induced neurotoxicity diminished the integrins β3, αV and induced the syndecan-1 and -3. Insulin pretreatment enhanced the phosphorylation of integrin-linked kinase and further induced the integrin and syndecan molecules. These findings suggest that insulin prevents MPP+-induced α-synuclein apoptosis through the activation of integrin and syndecan pathways in SH-SY5Y + RA cells.

1. Introduction

Parkinson's disease (PD) is the second most common age-related chronic neurodegenerative disease with dopaminergic neuronal cell death. α-synuclein (α-Syn; SNCA), is a major component of Lewy bodies in PD, and the aggregation of synucleins seems to be associated with neurodegenerative processes [1]. Autophagy is a regulated process that degrades and recycles cellular constituents of its own cytoplasm as a response to lack of nourishment, the effect of certain cytotoxins, and the presence of non-functional protein aggregates or in order to remove damaged organelles [2]. In addition, the cell-surface receptors that mediate cell-extracellular matrix (ECM) adhesion are primarily the integrins and syndecans. These are required for generating a physical link to the cytoskeleton, for force transduction and spatial control of the assembly of the adhesion signaling complex [3]. Integrin-linked kinase (ILK) known to bind to integrin β3 (β3; ITGB3) for its activation [4] and sequentially associates with integrin αV (αV; ITGAV). It was closely associated with synaptic function disorders, changes in plasticity, long-term inhibition, and regeneration of neurons [5]. Furthermore, the expression and distribution of syndecan-1 (SDC1) and syndecan-3 (SDC3) in neuronal cells may mediate the interactions with extracellular components that control the cell shape, adhesion, proliferation, and differentiation [6].

1-Methyl-4-phenylpyridinium ion (MPP+) is the active metabolite of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) Dopaminergic neurons taken up the MPP+ by dopamine and noradrenaline transporters, resulting in an inhibition of complex I of the mitochondrial election transport chain and forms reaction oxygen species (ROS). In our previous study, MPP+ can elevate

Abbreviations: α-Syn, SNCA, α-synuclein; Cox-2, cyclooxygenase-2; ILK, integrin-linked kinase; ITGAV, integrin αV; ITGB3, integrin β3; LDH, lactate dehydrogenase; MAP1LC3, microtubule-associated protein 1 light chain 3; MPP+, 1-Methyl-4-phenyl pyridinium; SDC1, syndecan-1; SDC3, syndecan-3.

* Corresponding author at: Department of Pharmacology and Toxicology, Metabolic Diseases Research Laboratory, School of Dentistry, Kyung Hee University, 26, Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea.
E-mail address: kimsj@khu.ac.kr (S.-J. Kim).

http://dx.doi.org/10.1016/j.neulet.2016.09.023
0304-3940/

oxidative stress by intracellular ROS and induce apoptotic cell death associated with changes in the Akt, insulin receptors (IR), insulin receptor substrates (IRS), and Bcl-2 family signaling pathways [7]. Insulin is highly enriched in the brain cortex, olfactory bulb, hippocampus, hypothalamus, and amygdala. Brain insulin signaling is required for the central glucose metabolism, feeding behavior, neurotransmission, learning and memory, and the maintenance of neural functions that could be helpful in the brain to avoid the development of neurodegenerative diseases [8]. Insulin pretreatment reduced the MPP^+-induced neurotoxicity in retinoic acid (RA)-differentiated SH-SY5Y cells and the hydrogen peroxide (H_2O_2)-induced oxidative damages in neuronal [9,10] and glial cells [11,12]. Therefore, in this study, we aimed to investigate the role of insulin on the expressions of α-Syn, autophagy, integrins, and syndecans in an experimental model of PD with MPP^+-induced neurotoxicity on RA-differentiated SH-SY5Y (SH-SY5Y + RA) neuroblastoma cells.

2. Materials and methods

2.1. Materials

Dulbecco's modified essential medium (DMEM), penicillin streptomycin (Pen Strep), trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Life Technologies (GIBCO, Grand Island, NY). Insulin was purchased from Roche Diagnostics (Mannheim, Germany). MPP^+-iodide, retinoic acid (RA), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), lactate dehydrogenase (LDH) *in vitro* toxicology assay kit (TOX-7) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). The PI3K inhibitor LY294002 and MEK inhibitor PD98059 were purchased from Abcam Biochemicals (Cambridge, UK). Plastic materials were purchased from SPL Life Science (SPL, Seoul, South Korea). All other reagents were from commercial suppliers and of the highest purity available.

2.2. Cell culture and treatment

The human neuroblastoma cell line SH-SY5Y (CRL-2266) was obtained from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% FBS, Pen Strep (100 U/ml; 100 μg/ml), and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. The cells were induced to differentiate into neuronal cells by the retinoic acid (RA, 10 μM). DMEM containing 1% FBS with fresh RA was replaced every 48 h and treated for 5-7 days. Confluent cultures were washed with phosphate-buffered saline (PBS), detached with 0.25% trypsin-EDTA solution, re-seeded as 5×10^5 cells/ml of DMEM containing 1% BSA and used for experiments after overnight incubation. RA-differentiated SH-SY5Y (SH-SY5Y + RA) cells were incubated with MPP^+ iodide in the absence (24 h) or presence of insulin (1 h pretreatment and 24 h co-incubation) and insulin alone (25 h) as explained in our previous study [7].

2.3. Lactate dehydrogenase (LDH) assay

The LDH *in vitro* toxicology assay kit (TOX-7, Sigma-Aldrich) was used to measure membrane viability as a function of the amount of cytoplasmic LDH released into the medium. The absorbance was measured at 490 nm using a TRIAD Series Multimode Detector (Dynex Technologies Inc., Chantilly, VA). The values were calculated as the relative intensity of absorbance compared with the control (untreated group) cells.

2.4. Western blotting analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 30 mM NaF, 2.5 mM NaPPi, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF) and centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were used as the total cell lysates. The protein concentrations were determined and 50 μg of protein were separated on 6–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to 0.45 μM Immobilon-P PVDF membranes (Millipore, Bellerica, MA). Membranes were incubated with primary antibodies of the phospho- or total ILK or Cox-2, α-synuclein, syndecan-1, syndecan-3, integrin αV, integrin β3 (Santa Cruz Biotechnology, Santa Cruz, CA), LC3A/B (Cell Signaling, Danvers, MA), and β-actin (Abcam, Cambridge, MA), and were detected by the luminol-based chemiluminescence (ECL) system (West-Q Pico Dura ECL Solution; GenDEPOT, Barker, TX) with a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). Densitometric analysis were performed using the ImageJ (National Institute of Health, Bethesda, MD) software.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies) and 2 μg was reverse transcribed for 60 min at 42 °C, followed at 94 °C for 15 min. The PCR consisting of denaturation at 94 °C for 30 s, primer annealing at Tm °C (Refer to Table 1) for 30 s, and extension at 72 °C for 30 s for 30 cycles was carried out. The amplified samples were loaded onto 1% agarose gels in Tris-Acetate-EDTA (TAE) buffer and visualized with ethidium bromide by a ChemiDoc XRS imaging system (Bio-Rad). The band intensity was quantified by ImageJ (National Institute of Health) densitometric analysis software. The primer pairs used for amplification are given in Table 1.

2.6. Statistical analysis

Data were analyzed using GraphPad Prism 5.0 software (La Jolla, CA), and were expressed as mean ± standard deviation (S.D.). The significance level of treatment effects was determined using a one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test between groups. A probability of <5% ($p < 0.05$) was considered to be statistically significant.

3. Results

3.1. Insulin on MPP^+-induced cell membrane integrity

From our previous study, the concentration causing about 68.05 ± 7.21% ($p < 0.001$) cell death after 24 h (500 μM MPP^+) was selected to test the protective effect of insulin (100 nM) in all subsequent experiments. Insulin was pretreated for 1 h and subsequently treated with 500 μM MPP^+ for 24 h [7]. As shown in Fig. 1A, the LDH released from cells exposed to 500 μM MPP^+ for 24 h was 253.8 ± 5.99% of the control value ($p < 0.001$). Cell membrane leakage was significantly decreased to 173.8 ± 6.59% ($p < 0.001$) when cells were pretreated with insulin.

3.2. Insulin on MPP^+-induced expressions on Cox-2 and LC3

To determine the neuroprotective effect of insulin against MPP^+-induced neurotoxicity, we performed the cyclooxygenase-2 (Cox-2). Results demonstrated an upregulation of 70–72 kDa Cox-2 protein expression (Fig. 1B; $p < 0.001$) after 24 h of MPP^+-induced toxicity. However, pretreatment with insulin (100 nM) significantly inhibited the Cox-2 expression caused by MPP^+. Moreover, the makeshift of microtubule-associated protein 1 light chain