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α-Synuclein and its neurotoxic fragment inhibit dopamine uptake into rat striatal synaptosomes Relationship to nitric oxide

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Abstract

α-Synuclein (ASN), a 140-amino acid protein, is richly expressed in presynaptic terminals in the central nervous system, where it plays a role in synaptic vesicle function. However, if it is altered and accumulated it is involved in neurodegeneration as Parkinson's disease (PD). ASN contained 35-amino acid domain known as non-amyloid beta component of Alzheimer's disease amyloid (NAC) that is probably responsible for its aggregation and toxicity. Up till now the role of ASN in dopaminergic system function and in pathogenesis of PD is unknown. The aim of this study was to determine the effect of brain aging and the role of ASN and NAC peptide on striatal dopamine transporter (DAT) function. The study was carried out using radiochemical and spectrofluorimetrical determination. It was found that DAT activity assessed by measuring [^{3}H]-dopamine (DA) uptake into striatal synaptosomes significantly decreased in 24-month-old rats comparing to 4-month-old. ASN and NAC peptide at 10 μM concentration inhibited DAT activity by 30%. Both molecules evoked intrasynaptosomal generation of reactive oxygen species measured by fluorogenic probe, 2′7′-dichlorofluorescin diacetate. In addition, ASN activated striatal cytosolic nitric oxide synthase (NOS) by 20%. Nitric oxide (NO) donor, sodium nitroprusside (SNP) (10 μM) and oxidative stress evoked by $FeCl_2$ (25 μM) reduced [^{3}H]DA uptake by 28 and 41%, respectively. Potent antioxidants: Trolox and 4-hydroxy-Tempo had no effect on DAT function but NOS inhibitor Nω-nitro-L-arginine (100 μM), prevented ASN-evoked DAT down-regulation. These data indicated an important role of ASN in alteration of DA synaptic homeostasis, probably by NO mediated DAT alteration.

Keywords: Alpha-synuclein; Dopamine transporter; Striatum; Nitric oxide

1. Introduction

Alpha-synuclein (ASN) is an ubiquitous protein that is especially abundant in the brain and has been postulated to play a central role in the pathogenesis of Parkinson's disease (PD), dementia with Lewy bodies (DLB), neurodegeneration with brain iron accumulation type 1 (NBIA-1), and other age-related neurodegenerative disorders which collectively are termed synucleinopathies. The link between neurodegenerative disease and ASN, however, was first reported for Alzheimer's disease (AD) when the small peptide termed 'NAC' was purified from AD brain, leading to the cloning of the non-amyloid beta component precursor protein (NACP, Ueda et al., 1993), now known to be ASN. More recent studies revealed that NAC peptide, which is a 35-amino acid fragment of ASN (residues 61–95) could be responsible for its aggregation and toxicity (Bodles et al., 2000; Giasson et al., 2001). Accumulating data showed close relationship between ASN and dopaminergic neurotransmission involved in the control of locomotor activity and regulation of goal-oriented behavior and reward (Schultz, 2002). It is suggested that ASN in native soluble form modulates dopamine (DA) biosynthesis, storage into vesicles and release, as well as the reuptake of this neurotransmitter (Perez and Hastings, 2004; Yu et al., 2005). Aggregation of ASN into protofibrils causes loss in its normal functions and dysregulation of dopaminergic neurotransmission. Increased level of DA could activate free radicals formation. Enzymatic deamination of DA by monoamine oxidases (MAO) leads to the production of the non-toxic 3,4-dihydroxyphenylacetic acid

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(DOPAC) and hydrogen peroxide (H_2O_2) (Maker et al., 1981). In turn, H_2O_2 can be converted to highly toxic hydroxyl radicals. DA can also undergo spontaneous auto-oxidation into toxic and reactive DA-quinones, superoxide free radicals and H_2O_2 (Graham et al., 1978). On the other hand, the reactive oxygen species (ROS) produced by DA metabolism could promote aggregation and toxicity of ASN (Conway et al., 2001; Xu et al., 2002). It was reported by Lee et al. (2001) that DA transporter (DAT) activity is stimulated by ASN. However, Wersinger et al. (2003) observed inhibitory effect. The last data showed that ASN is not only accumulated intracellular but also can acts extracellularly (Seo et al., 2002; Zhang et al., 2005; Sung et al., 2005). Moreover, full-length ASN was found in cerebrospinal fluid (CSF) from PD patients and also in age-related control (Borghi et al., 2000). These all results indicated that ASN could be present extracellularly and exert toxicity. The aim of our study was to investigate the role of ASN and its neurotoxic fragment NAC on DA uptake into striatal synaptosomes. Moreover, the relationship between DA uptake and ASN alteration in aged brain was evaluated.

2. Materials and methods

2.1. Materials

The following products were used: [2,5,6-^{3}H]DA, 8.50 Ci/mmol (Amersham Biosciences, UK), α-synuclein (rPeptide, USA). All other chemicals were purchased from Sigma.

2.2. Animals

Male Wistar rats adult (4-month-old) and old (24-month-old) obtained from the Animal Breeding House of the Medical Research Centre, Warsaw, Poland were used in all experiments. Animals were kept behind the barrier system and were specific pathogen free (SPF). The Institutional Ethics Committee accepted the research project. After animals' decapitation, brains were rapidly removed and the striatum dissected on ice-cooled dish according to Glowinski and Iversen (1966).

2.3. Preparation of striatal synaptosomal fraction

Synaptosomes were prepared as previously described by Morel et al. (1998), with slight modifications. Striatum were homogenized in 10 vol. of 0.32 M sucrose with 10 mM Tris–HCl and 1 mM EDTA, pH 7.4 using 12 up- and down-strokes of glass–glass Dounce homogenizer. The homogenates were then centrifuged at 1000 × *g* for 10 min at 4 °C to obtain a crude nuclear pellet. Supernatants were stored at 4 °C, and the pellet was re-suspended in 10 vol. of homogenizing buffer and centrifuged for 10 min at 1000 × *g*. The two supernatants were pooled and centrifuged at 17,500 × *g* for 30 min at 4 °C. The resulting supernatant was discarded and the synaptosomal fraction was re-suspended in ice-cold Krebs–Ringer buffer, pH 7.6 (NaCl, 120 mM; KCl, 5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; glucose, 10 mM). Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.4. Preparation of striatal plasma membranes and cytosol

Striatum was homogenized using 14 up- and down-strokes of glass–glass Dounce homogenizer in 0.32 M sucrose containing 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT) and protease inhibitors (Roche Diagnostics GmbH, Germany). The homogenate was then centrifuged at 900 × *g* for 3 min at 4 °C. Resulting pellet (P_1) was discarded and the supernatant (S_1) was centrifuged at 100,000 × *g* for 60 min at 4 °C to obtain cytosolic fraction (S_2). Plasma membranes (PM) were obtained as described previously (Zambrzycka et al., 2000).

2.5. Measurement of DA uptake

DA uptake was assayed as previously described by Morel et al. (1998), using tritiated ([^{3}H]DA) and non-labeled DA to obtain a final concentration 400 nM. Synaptosomes were pre-incubated for 30 min at 37 °C in 2 ml Krebs–Ringer buffer. Then $CaCl_2$ and pargyline was added to obtain 2 mM and 100 μM concentration, respectively. ASN (10 μM), NAC (10 μM) or other investigated compounds were subsequently added to the appropriate tube and pre-incubation was prolonged for the other 30 min at 37 °C. Pre-incubations were performed in the presence and absence of nomifensine (10 μM), a specific DAT inhibitor, in order to check the specific DA uptake. The assay was initiated by addition of [^{3}H]DA to synaptosomes (60–100 μg protein/assay). After 40-s, 2-min and 5-min of DA uptake at 37 °C the reaction was stopped by addition of 3 ml of ice-cold Krebs–Ringer buffer, followed by vacuum filtration through Whatman GF/C filters. Each tube was rinsed 2× 3 ml of ice-cold Krebs–Ringer buffer. The filters were placed in scintillation vials containing 7 ml of scintillator and stored at room temperature overnight. Radioactivity was determined by liquid scintillation spectrometry (Wallac 1409, LKB). Specific uptake was calculated by subtracting non-specific uptake, determined in the presence of nomifensine, from total uptake. In each experiment, assays were performed in duplicate.

2.6. Determination of oxidative stress

Intrasynaptosomal generation of ROS was measured using fluorogenic probe, 2′7′-dichlorofluorescin diacetate (also known as 2′7′-didichlorodihydrofluorescein diacetate; DCFH-DA). DCFH-DA is intracellularly/intrasynaptosomal deacetylated to 2′7′-dichlorofluorescin (DCFH) and then oxidized by hydrogen peroxide to a fluorescent compound, 2′7′-dichlorofluorescein (DCF). Freshly isolated synaptosomes suspended in Locke's buffer, pH 7.2 (NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2.3 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 3.6 mM; Hepes, 5 mM; glucose, 5 mM) were mixed with DCFH-DA at 15 μM concentration and incubated in a water shaking bath in the presence of ASN and NAC at 10 or 25 μM $FeCl_2$ in the dark for 30 min at 37 °C under carbogen. The concentration of DCF was measured by a fluorescence spectrophotometer (ELS 50B, Perkin-Elmer) with excitation at 488 nm and emission at 530 nm.

2.7. Lipid peroxidation assay

Lipid peroxidation was evaluated by determining the malondialdehyde using thiobarbituric acid assay (Asakawa and Matsushita, 1980). Synaptosomes suspended in Krebs–Ringer buffer, pH 7.4 at protein concentrations of approximately 1.0 mg/ml were incubated for 30 min at 37 °C with 10 μM SNP or $FeCl_2$ (25 μM)/ascorbic acid (250 μM). After incubation, 1 ml of 30% trichloroacetic acid (TCA), 0.1 ml of 5 M HCl and 1 ml of 0.75% thiobarbituric acid were added to the vials. The tubes were capped, the mixtures were heated at 100 °C for 15 min in a boiling water bath, and then centrifuged at 1000 × *g* for 10 min. Thiobarbituric acid reactive substances (TBARS) were measured in supernatant at 535 nm.

2.8. Nitric oxide synthase assay

Nitric oxide synthase activity was assayed in striatal cytosol as described by Strosznajder and Chalimoniuk (1996). Cytosol (about 350 μg/150 μl) was pre-incubated for 30 min at 37 °C in the presence and absence (control) of 10 μM ASN. Then, 150 μl of assay mixture containing 50 mM Tris–HCl, pH 7.4, 100 μM arginine L-[^{14}C (U)] 0.1 μCi (sp. act. 360 mCi/mmol), 2 mM $CaCl_2$, 1 μM calmodulin, 15 μM FAD, 10 μM tetrahydrobiopterin (BH_4), 1 mM NADPH, 1 mM EDTA and 1 mM DTT were added to each vial. The mixture was incubated for 20 min at 37 °C. Reaction was terminated by addition of 1ml buffer containing 100 mM Tris–HCl, pH 5.5 with 10 mM EDTA. Then the mixture (500 μl) was passed through 0.5 ml of Dowex™ 50WX-8 columns (Na^+ form) and [^{14}C]L-citrulline was eluted with H_2O (2× 1 ml). Radioactivity of eluted [^{14}C]L-citrulline was determined using Bray's solution in LKB Wallac 1409 counter.