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Alpha-synuclein decreases arachidonic acid incorporation into rat striatal synaptoneurosomes

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Abstract

In the present study we investigated the role of alpha-synuclein (ASN) and its fragment NAC in arachidonic acid (AA) turnover in the rat brain. Our data indicated that ASN (10 μM) inhibited [^{3}H]AA incorporation into phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) together with phosphatidic acid (PA) by 13%, 27% and 38%, respectively. NAC (10 μM) reduced [^{3}H]AA incorporation into PC and PA + PS by 17% and 34%, respectively. Because ASN and NAC lower the level of AA-CoA we suggest that inhibition of AA-CoA synthase is responsible for disturbed [^{3}H]AA incorporation. In conclusion, ASN and NAC modulate phospholipid compositions in plasma membranes and affect synaptic endings function.

Key words: *striatum, alpha-synuclein, non-amyloid beta component of Alzheimer's disease amyloid, arachidonic acid*

Introduction

Alpha-synuclein (ASN) is a 140-amino acid soluble protein expressed in the brain and has been postulated to play an important 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 physiological role and the mechanism of ASN action during neurodegenerative diseases still remain unclear. More recent studies revealed that the non-amyloid beta component of Alzheimer's disease amyloid (NAC), which is a 35-amino acid fragment of ASN (residues 61-95), could be responsible for its aggregation and toxicity [5].

ASN concentrated at the synaptic terminals could be involved in membrane structure and function, synaptic vesicle formation, neurotransmitter release and vesicle pool size [3]. Recent data indicated that ASN is present in the extracellular space [24]. ASN may interact with membrane microdomains that may be necessary for its localization in synaptic terminals [10]. *In vitro* studies indicated that ASN influences phospholipid composition through inhibition of phospholipase D_2 [12], which may affect signal transduction and vesicle trafficking. Moreover, ASN alters phospholipase C beta (PLCβ) [16] and downregulates expression of phospholipase A_2 (PLA_2) and long chain fatty acid CoA synthetase in *Drosophila* [19]. In addition, it binds to and inhibits protein kinase C (PKC) [17]. Recently, the interaction of ASN with polyunsaturated fatty acids (PUFA) was investigated

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[7,11,15,20,21]. However, the role of ASN in brain arachidonic acid (AA) turnover is still not known. This fatty acid is a precursor in the production of eicosanoids: the prostaglandins, thromboxanes, prostacyclins and the leukotrienes. During this AA metabolism superoxide anion (O_2^-) is liberated. Therefore, AA could be cytotoxic, causing apoptosis and/or necrosis [18]. The aim of our study was to investigate the role of ASN and its neurotoxic fragment NAC on AA incorporation and release in striatal and cortical synaptoneurosomes. Moreover, the effect of beta-synuclein (BSN) on these processes was evaluated.

Material and Methods

Materials

[^{3}H]AA, 55 mCi/mmol was purchased from NEN, USA, ASN and BSN was obtained from rPeptide, USA, TLC plates from Merck AG, Germany. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Preparation of synaptoneurosomal fraction

All experiments on animals were accepted by the Polish National Ethics Committee and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Adult (4-month-old, 250-300 g) male Wistar rats were sacrificed and the brains were rapidly removed. The synaptoneurosomal fraction was obtained from the rat brain cortex and striatum as described previously [23]. The slices were prepared manually with a cooled razor blade and homogenized by hand (5 strokes) in 7 ml of Ca^{2+}-free Krebs-Henseleit bicarbonate buffer (KREBS: 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose equilibrated with O_2/CO_2 (95/5 v/vl)), pH 7.4, using a Dounce-type glass homogenizer. After dilution to 35 ml of KREBS buffer and centrifugation at 1100 × g for 15 min, the pellet containing the synaptoneurosomal fraction was resuspended in KREBS buffer and preincubated at 37°C for 30 min under O_2/CO_2 atmosphere (95/5, v/vl) and then used for the assay of [^{3}H]AA incorporation.

Determination of oxidative stress

Intrasynaptosomal generation of ROS was measured using fluorogenic probe, 2'7'-dichlorofluorescin diacetate (also known as 2'7'-dichlorodihydrofluorescein diacetate; DCFH-DA). DCFH-DA is intracellularly/intrasynaptosomally deacetylated to 2'7'-dichlorodihydrofluorescein (DCFH) and then oxidized by hydrogen peroxide to a fluorescent compound, 2'7'-dichlorofluorescein (DCF). Freshly isolated synaptoneurosomes suspended in Locke's buffer, pH 7.2 (in mM: NaCl 154, KCl 5.6, $CaCl_2$ 2.3, $MgCl_2$ 1.0, $NaHCO_3$ 3.6, HEPES 5, and glucose 5) were mixed with DCFH-DA at 15 µM concentration and incubated in a shaking water bath in the presence of soluble and aggregated NAC peptide at 10 µM or $FeCl_2$ /ascorbate (25 µM/250 µM) in the dark for 30, 60 and 120 min at 37°C under carbogen. The concentration of DCF was measured by a fluorescence spectrophotometer (LS 50B, Perkin-Elmer) with excitation at 488 nm and emission at 530 nm.

Assay of [^{3}H]AA incorporation into phospholipids

The [^{3}H]AA incorporation into phospholipids in cortical and striatal synaptoneurosomes was assayed in the presence of ASN, NAC peptide and BSN, each compound at 10 µM concentration. Tubes containing 0.7 mg of synaptoneurosomal protein, 2.0 µCi [^{3}H]AA, (specific activity 55 mCi/mmol), 2.5 mM ATP, 10 mM $MgCl_2$, 0.1 mM CoA, 0.3 mM DTT and appropriate agents (ASN, NAC, BSN) were incubated during 20 min at 37°C in a shaking water bath. Then, the reaction was stopped by addition of 2 vol of chloroform/methanol mixture (1/2, v/v) and the lipids were extracted according to Bligh and Dyer [4].

Assay of the level of [^{3}H]arachidonoyl-CoA radioactivity

The upper water phase obtained after lipid extraction contained [^{3}H]AA-CoA and also some pool of [^{3}H]AA, which was removed after the washing procedure. This water phase was washed three times with 1 ml of n-heptane, each time centrifuged at 900 × g for 5 min. Then, a 0.5 ml aliquot of the water phase was taken for measurement of [^{3}H]AA-CoA radioactivity using Bray's scintillation fluid. Radioactivity of the samples was measured in an LKB 1409 Wallac scintillation counter.

Assay of [^{3}H]AA release

Before determination of [^{3}H]AA release, arachidonic acid incorporation into synaptoneurosomes was carried out in an incubation system which contained 2.0 µCi [^{3}H]AA (specific activity 55 mCi/mmol), 2.5 mM ATP, 10 mM $MgCl_2$, 0.1 mM CoA and 0.3 mM DTT as described above but in the presence of 10 mg of synaptoneurosomal protein and in a total volume of 5 ml. The incubation was carried out at 37°C for 30 min in a shaking water bath. Then, prelabelled synaptoneurosomes were washed twice with KREBS containing 0.1% BSA (fatty acid free) by centrifugation at 1100 × g for 15 min and once with the same buffer without addition of BSA.

For determination of [^{3}H]AA release, the [^{3}H]AA--prelabelled synaptoneurosomes were subsequently resuspended in KREBS buffer to obtain the protein concentration of about 2 mg/ml. Subsequently, an aliquot amount of synaptoneurosomal suspension, 2 mM $CaCl_2$ and the other compounds, applied depending on experimental conditions, were added to incubation vials to a final volume of 0.2 ml. Incubation was carried out for 15 min at 37°C in a shaking water bath. Reaction was terminated by addition of 2 vol of chloroform/methanol mixture (1/2, v/v) and lipids were extracted according to Bligh and Dyer [4]. The lower chloroform phase was collected and evaporated to dryness under nitrogen.

Separation of lipids and determination of AA release and its incorporation

For determination of the AA incorporation into the lipids, the lipid extract was separated on silica gel TLC plates using a solvent system consisting of chloroform/methanol/4M ammonium hydroxide (9/7/2, v/v/v). The lipid spots, visualized in iodine vapour, and those corresponding to PE, PC, PI and other lipid standards, were scraped into scintillation vials with 8 ml of Bray's fluid and the radioactivity was estimated in an LKB Wallac 1409 scintillation counter.

In the case of measurement of AA release, the separation of AA from the other lipids was carried out using chloroform/acetone (96/4, v/v) as a mobile phase. After identification of lipids in iodine vapour, AA and total phospholipids spots were scraped off the plates into scintillation vials with 8 ml of Bray's fluid. Radioactivity of the samples was measured in an LKB 1409 Wallac scintillation counter.

Protein content was estimated according to Lowry et al. [14].

Assay of the role of oxidative stress on ASN liberation from synaptoneurosomes into extracellular space

A 0.5 ml aliquot of synaptoneurosomes in KREBS buffer with 1 mM $CaCl_2$ was incubated at 37°C for 6 h with $FeCl_2$ /ascorbate (25 µM/250 µM), H_2O_2 (500 µM) and SNP (1 mM) and without these compounds (control). Then synaptoneurosomal suspension was centrifuged at 13000 × g for 20 min. The obtained supernatant (±500 µl) was lyophilized, then resolved in 20 ml of KREBS, diluted 1:1 with 2 × electrophoresis sample buffer (2×SB) and incubated at 95°C for 5 min. The pellet containing synaptoneurosomes was also mixed with 2×SB. Then, in supernatants and pellet ASN level was measured by Western blot analysis as described previously [2].

Statistical analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by a Newman--Keuls post-hoc test. Differences among groups were considered significant if the probability of error was less than 5%. The data represent mean ± S.E.M.

Results

Our results indicate that ASN is liberated from synaptoneurosomes into extracellular space during oxidative stress evoked by $FeCl_2$ /ascorbate (25 µM/ /250 µM), H_2O_2 (500 µM) and nitric oxide (NO) donor sodium nitroprusside (SNP, 1 mM) (Fig. 1). Then we evaluated the effect of extracellular ASN, its neurotoxic fragment NAC and BSN (each at 10 µM concentration) on the [^{3}H]AA incorporation into striatal and cortical synaptoneurosomes. During the 20-min period of incubation ASN reduced [^{3}H]AA incorporation into phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) together with phosphatidic acid (PA) by 13%, 27% and 38%, respectively in striatal synaptoneurosomes (Fig. 2). NAC peptide in the same experimental conditions inhibited [^{3}H]AA incorporation into PC and PS + PA by 17% and 34%, respectively (Fig. 2). Incorporation of [^{3}H]AA into phosphatidylinositol (PI) was not alte-

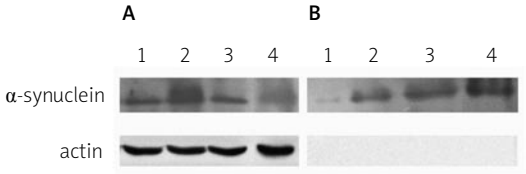

Fig. 1. Oxidative stress caused ASN liberation from synaptoneurosomes into extracellular space. Representative Western blot from 3 independent experiments. (A) Synaptoneurosomal fraction; (B) extracellular buffer. Lanes: 1 – control, 2 – SNP (1 mM), 3 – H_2O_2 (500 µM), 4 – $FeCl_2$/Asc. (25 µM/250 µM)

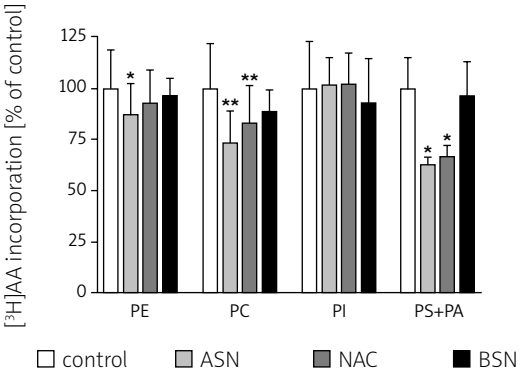

Fig. 2. The effect of ASN, NAC and BSN on [^{3}H]AA incorporation into phospholipids. Assay of [^{3}H]AA incorporation into synaptoneurosomal phospholipids from striatum was determined as described in Material and Methods. Data represent the mean value ± S.E.M. from 4-5 animals; each experiment was carried out in triplicate. Significance of data was determined with one-way ANOVA followed by Newman-Keuls test, * $p<0.05$, ** $p<0.01$ versus control

red. Moreover, BSN had no effect on [^{3}H]AA incorporation into all investigated phospholipids. In cortical synaptoneurosomes exclusively NAC peptide reduced [^{3}H]AA incorporation into PC by 23% (data not shown). Our previous study showed that α-synuclein significantly activated nitric oxide synthase and free radical generation [1]. The data presented in this study also demonstrate that both soluble and aggregated NAC induced time-dependent free radical formation (Fig. 3). The effect of this peptide was similar to the effect of iron ions. We also indicated previously that oxidative stress evoked by AA metabolites and nitric oxide (NO) donor sodium nitroprusside (SNP, 1 mM) caused significant decrease of [^{3}H]AA incorporation into cortical synaptoneurosomes [8,22]. Moreover it was also reported that free radicals significantly affected phospholipid metabolism [25] and neurotransmission processes in the brain [6]. To determine whether reactive oxygen species might be involved in inhibition of [^{3}H]AA incorporation evoked by α-synuclein, the effect of antioxidant Trolox (1 mM) was evaluated. The results showed that this compound had no effect on [^{3}H]AA incorporation decreased by ASN (data not shown). To evaluate which enzyme could be responsible for inhibition of [^{3}H]AA incorporation into striatal phospholipids the level of [^{3}H]AA-CoA was measured in the same experimental conditions. Our data indicate that ASN and NAC reduced [^{3}H]AA-CoA formation in the striatum by about 10% without an effect of BSN (Fig. 4). AA release from [^{3}H]AA prelabelled cortical and striatal synaptoneurosomes was not modulated by ASN, NAC and BSN (data not shown).

Discussion

Our study showed for the first time that oxidative stress conditions led to ASN release from synaptoneurosomes. Extracellularly liberated ASN can exert a cytotoxic effect on neighbouring cells [26] or it could be cleaved to NAC by extracellular peptidases [24]. Moreover, we indicated that extracellular ASN and its neurotoxic fragment NAC inhibited [^{3}H]AA incorporation into phospholipids without changes in its release by PLA_2, whereas non-toxic BSN did not modulate AA turnover. Since ASN affected exclusively striatal synaptoneurosomes, we suggest that this brain structure is more sensitive to this protein than the cortex. ASN-evoked inhibition of AA incorporation into phospholipids could be responsible for increased level of intracellular free AA. This fatty acid plays an important role in the formation of oxygen free radicals including O_2^-. Superoxide anion is produced by cyclooxygenases and lipoxygenases that metabolize AA to eicosanoids [13]. Overproduction of reactive oxygen species is suggested to have an important role in PD and other synucleinopathies. Free radicals and also AA itself could promote the formation of highly soluble oligomers of ASN, which precedes the insoluble toxic aggregates. Sharon et

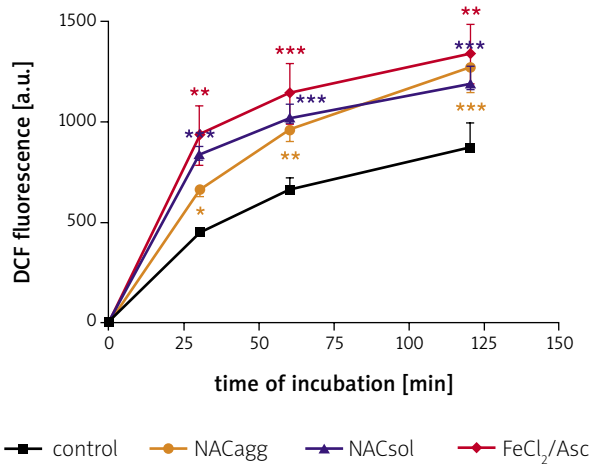

Fig. 3. Time-dependent free radical generation induced by soluble (NACsol) and aggregated (NACagg) NAC peptide. Assay of intrasynaptosomal generation of ROS was determined as described in Material and Methods. Data represent the mean value ± S.E.M. for four separate experiments. Significance of data was determined with one-way ANOVA followed by Newman-Keuls test, * p<0.05, ** p<0.01, *** p<0.001 versus control

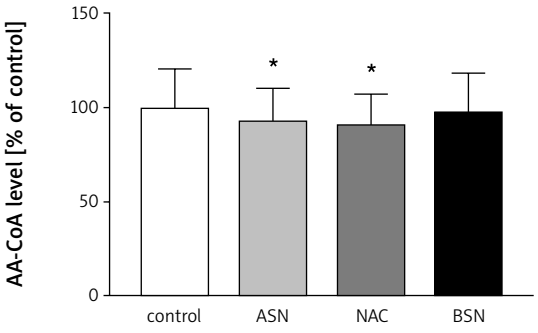

Fig. 4. ASN and NAC peptide decrease [^{3}H]AA-CoA level in striatal synaptoneurosomes. Assay of the level of [^{3}H]AA-CoA was determined as described in Material and Methods. Data represent the mean value ± S.E.M. from 4-5 animals; each experiment was carried out in triplicate. Significance of data was determined with one-way ANOVA followed by Newman-Keuls test, * p<0.05, versus control

al. [20] suggested that the increased levels of soluble ASN oligomers in PD and DLB brains could be associated with changes in the composition of endogenous brain fatty acids. Moreover, impaired membrane polyunsaturated fatty acid (PUFA) composition disturbs membrane fluidity. As a result, diverse cellular functions such as receptor signalling, membrane transporter activation, ion channel conductance and neurotransmitter release are affected. Other membrane properties that are known to be affected by PUFA composition are membrane thickness, deformability and curvature. ASN through AA metabolism alteration could affect one or more of these membrane properties. Previous data indicated reduced PUFA levels in parkinsonian substantia nigra compared to control tissue [9]. However, it was not characterized which fatty acid was changed in PD. Sharon et al. [20] showed an increase in docosahexaenoic and docosatetraenoic acid level in the cytosolic fraction from PD and DLB brains without changes in AA level. Our results indicated lower turnover of AA with significant inhibition of its incorporation in the presence of ASN.

AA incorporation into phospholipids is regulated by two concerted enzymatic activities: AA-CoA synthetase and AA-CoA:lysophospholipid transferase. The first one catalyzes the ATP- and Mg_{2+}-dependent formation of AA-CoA, using fatty acid and reduced CoA as substrates. Next, the activated fatty acid is incorporated into lysophospholipid by AA-CoA:lysophospholipid transferase. In the present study we asked which of these enzymes could be responsible for reduced AA incorporation evoked by ASN. The finding that ASN and its fragment NAC reduced the level of AA-CoA indicated that lower [^{3}H]AA incorporation could be due to inhibition of AA-CoA synthetase. Since antioxidant Trolox did not prevent ASN-evoked suppression of [^{3}H]AA incorporation, we suggest that free radicals are not involved in this ASN action.

In conclusion, these data indicate that ASN modulates phospholipid composition in plasma membranes and in this way may affect synaptic endings function. AA might be an important factor in pathomechanism of PD and other neurodegenerative disorders and prevention of this free fatty acid accumulation might be a useful therapeutic strategy for the treatment of PD.

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