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fractions.

A: Effects of melanin species on SH levels in the soluble and precipitate fraction of mitochondria. Mitochondria prepared from SH-SY5Y cells were incubated without (**I**) or with 25 µg/ml NM (**II**), P-K NM (**III**), DAM (**IV**), Cys-DAM (**V**) and 250 µM dopamine (**VI**) for 2 h at 37°C, then subjected to fractionation into the supernatant fraction and the sediment. SH levels were measured with Thiol Assay Kit and expressed as nmol/fraction. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.01$.

B: Effects of melanin on SH levels in cytosol and mitochondria fractions. The wild cells were treated with 25 µg/ml of NM and DAM at 37°C for 2 h and subjected to subcellular fractionation. Mitochondrial and cytosol fraction were prepared for GSH analysis by GSH reductase-dependent recycle method. **I**, Control. **II** and **III**, cells treated with NM and DAM. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.01$ from control.

Figure 6. Effects of anti-oxidants and NADPH on the NM-induced apoptosis and SH increase.

A: Effects of antioxidants on cell death induced by NM. The wild cells were treated without (**I**, control) or with NM alone (**II**) or NM in the presence of DFX (**III**, 1 μ M), SOD (**IV**, 100 unit/ml), catalase (**V**, 300 u/ml) and EGCG (**VI**, 10 μ M) for 16 h at 37°C. Live cells were quantified using calcein staining. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.05$ from control. #, Difference from NM-treated cells, $p < 0.05$.

B: Effects of antioxidants on SH levels in mitochondria. Mitochondria were treated without or with NM in the absence (**I**, control) or presence of DFX (**II**, 1 μ M), SOD (**III**, 100 unit/ml), catalase (**V**, 300 unit/ml) and EGCG (**VI**, 10 μ M) for 2 h at 37°C. SH contents were measured fluorometrically using the Thiol Assay Kit. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.05$. #, Difference from NM-treated cells, $p < 0.05$.

C: Effects of NADPH on SH contents in melanin-treated mitochondria. Mitochondria were treated without (Control) or with 25 μ g/ml NM or DAM at 37°C for 2 h, in the

absence (-) or presence (+) of 1 mM NADPH. SH was quantified with the Thiol Assay Kit. The column and bar represent the mean and SD of the quadruplicate measurements of two experiments. *, Difference from control, $P < 0.01$. #, Difference from NM-treated cells, $p < 0.05$.

Figure 7. Effects of NM on S-glutathionylated protein in mitochondria. Mitochondria were prepared from the wild cells and treated without (I, control), or with 10 $\mu\text{g/ml}$ NM (II), P-K NM (III), DAM (IV), Cys-DAM (V) or 100 μM dopamine (VI), at 37°C for 2 h. The samples were washed with PBS, and subjected to SDS-PAGE under non-reducing (A) and reducing conditions (B). S-Glutathionylated protein (PrS-SG) was visualized by use of polyclonal anti-GSH antibody. Complex I and III were detected with the antibody against complex I and III, respectively. The left line of each gel represents the protein markers with molecular mass with 250, 150, 100, 75, 50, 37, 20, 15 and 10 kDa from the top,

Figure 1.

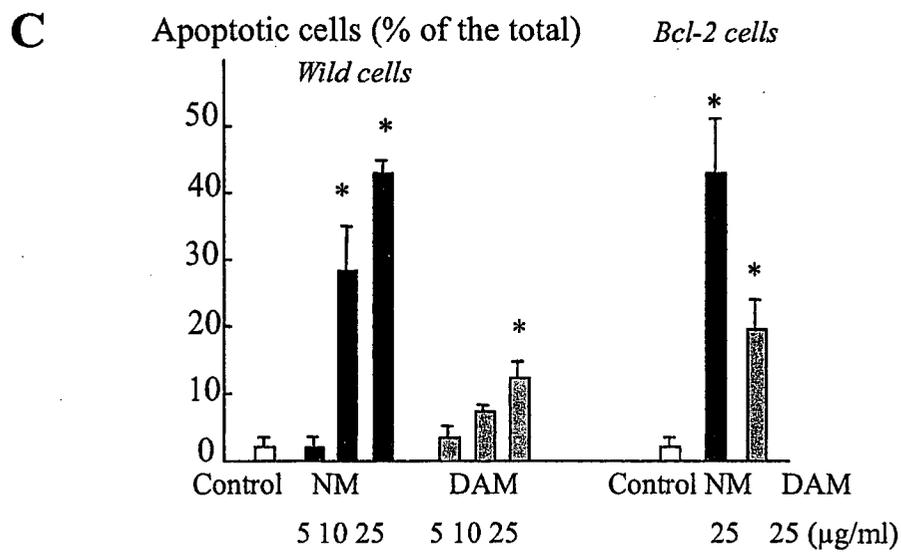
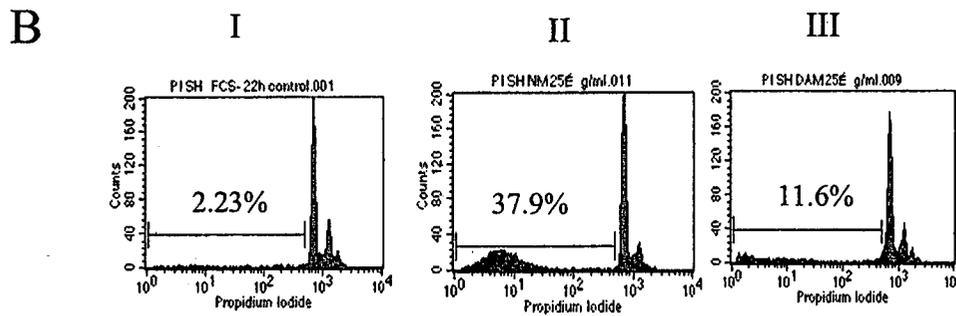
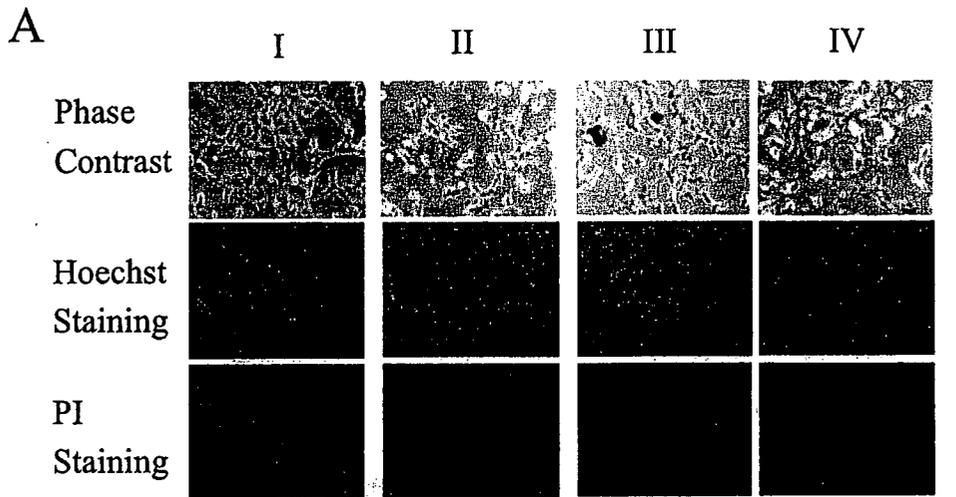
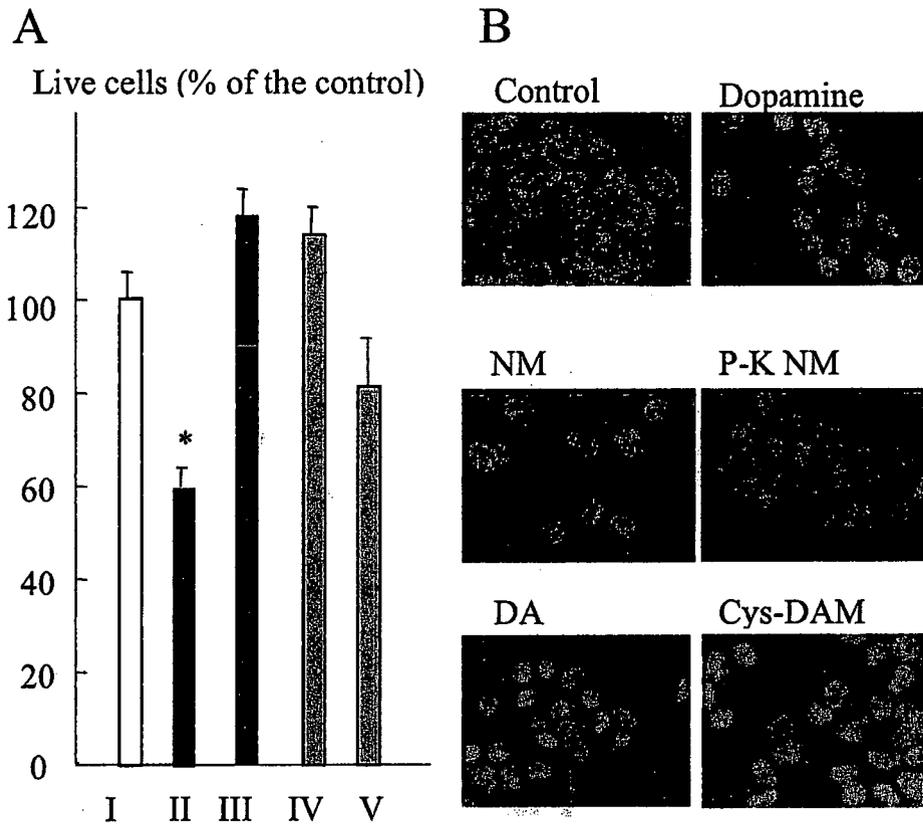
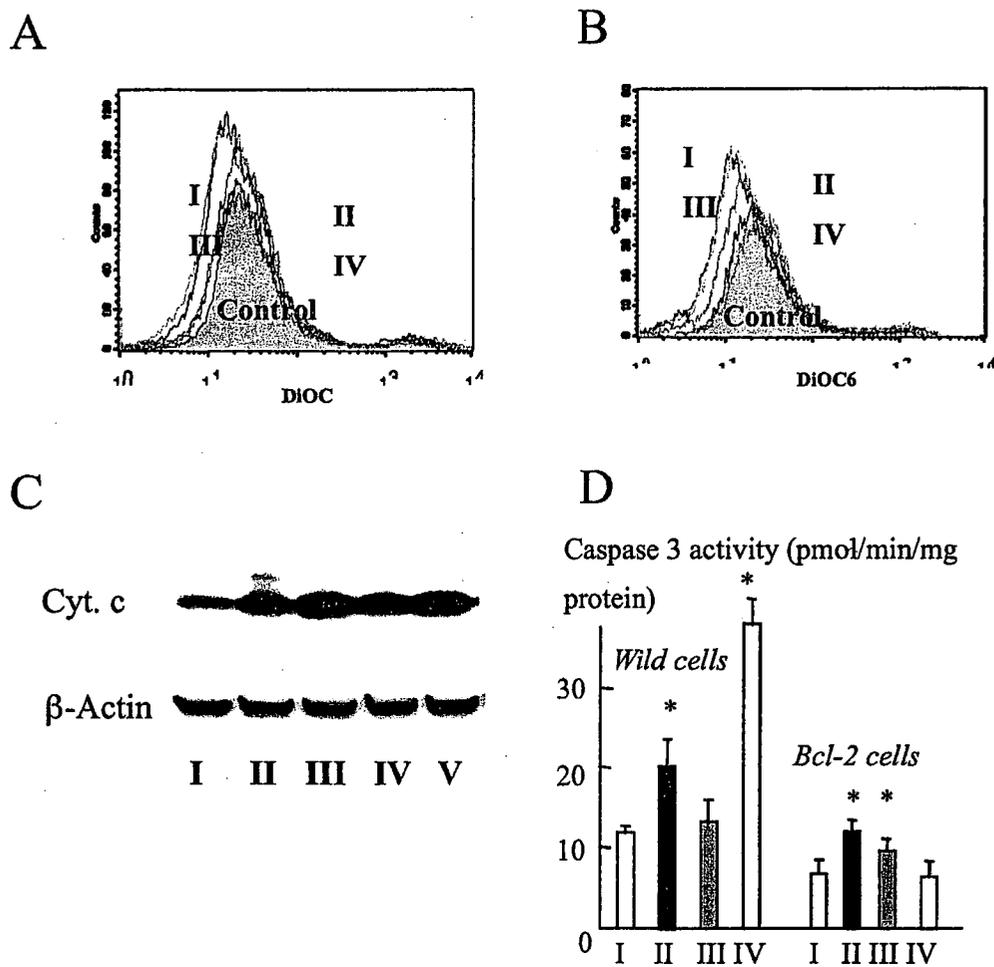


Figure 2.



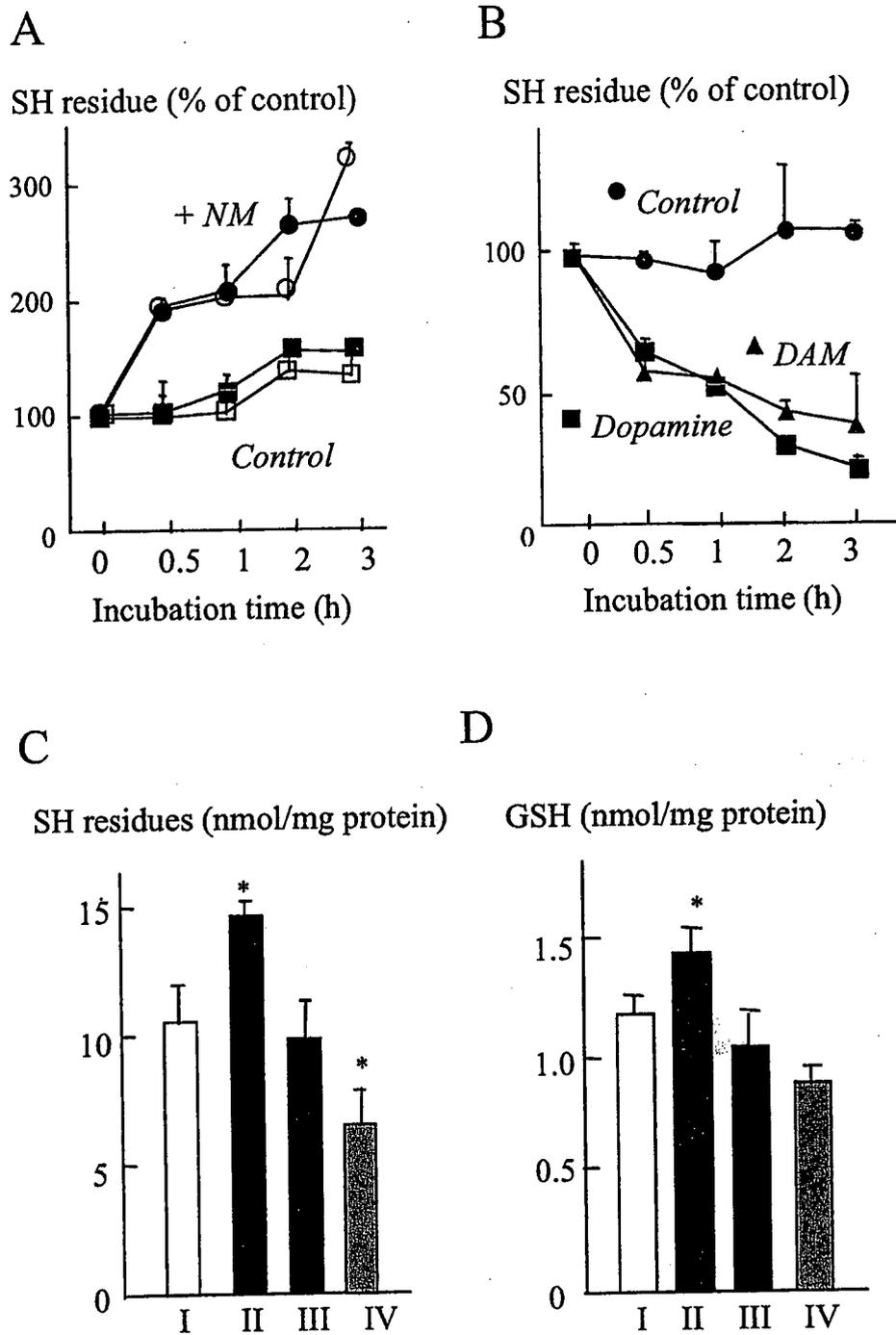
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Figure 3.



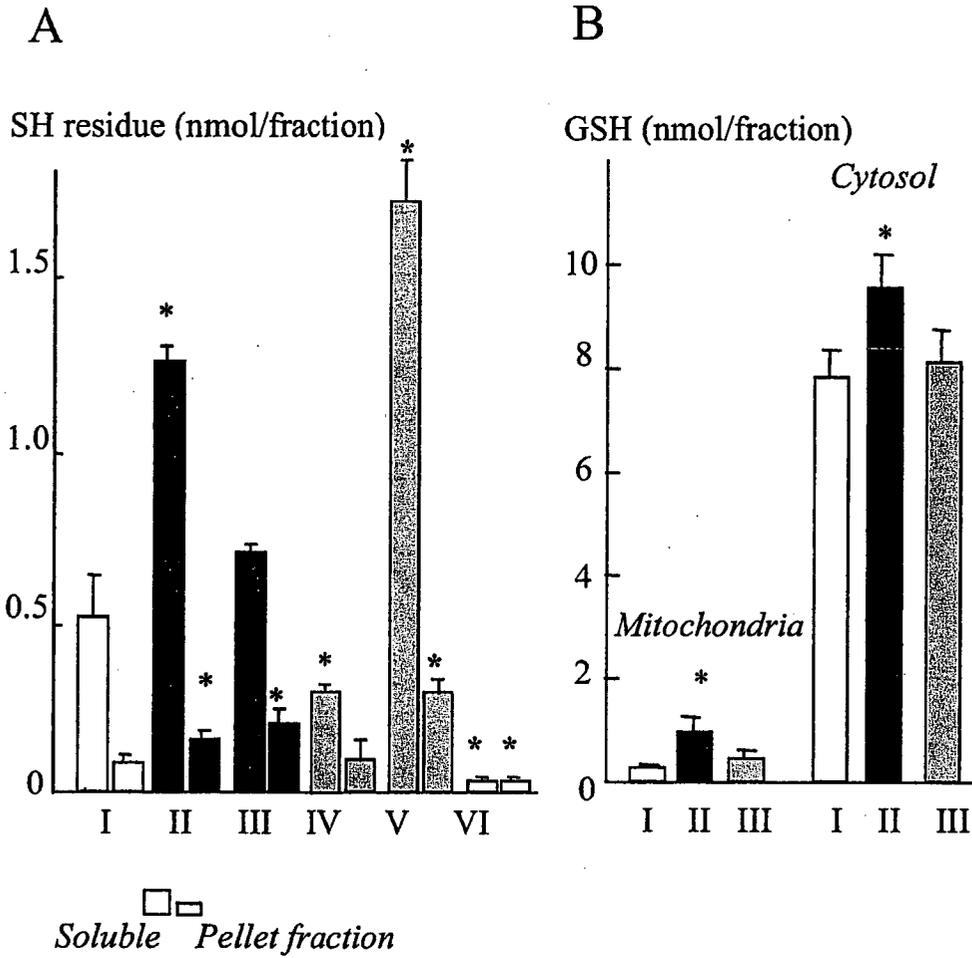
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Figure 5.



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In parkinsonian substantia nigra, α -synuclein is modified by acrolein, a lipid-peroxidation product, and accumulates in the dopamine neurons with inhibition of proteasome activity

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Summary. α -Synuclein (α SYN) plays a central role in the neural degeneration of Parkinson's disease (PD) through its conformational change. In PD, α SYN, released from the membrane, accumulates in the cytoplasm and forms Lewy body. However, the mechanism behind the translocation and conformational change of α SYN leading to the cell death has not been well elucidated. This paper reports that in the dopamine neurons of the substantia nigra containing neuromelanin from PD patients, α SYN was modified with acrolein (ACR), an aldehyde product of lipid peroxidation. Histopathological observation confirmed the co-localization of protein immunoreactive to anti- α SYN and ACR antibody. By Western blot analyses of samples precipitated with either anti- α SYN or anti-ACR antibody, increase in ACR-modified α SYN was confirmed in PD brain. Modification of recombinant α SYN by ACR enhanced its oligomerization, and at higher ACR concentrations α SYN was fragmented and polymerized forming a smear pattern in SDS-PAGE. ACR reduced 20S proteasome activity through the direct modification of the proteasome proteins and the production of polymerized ACR-modified proteins, which inhibited proteasome activity *in vitro*. These results suggest that ACR may initiate vicious cycle of modification and aggregation of proteins, including α SYN, and impaired proteolysis system, to cause neuronal death in PD.

Keywords: Acrolein; α -synuclein; Parkinson's disease; protein aggregation; dopamine neuron; proteasome

Abbreviations

ACR acrolein
 α SYN α -synuclein
HNE 4-hydroxy-2-nonenal
PD Parkinson's disease

RNS reactive nitrogen species
ROS reactive oxygen species
UPS ubiquitin-proteasome system

Introduction

Accumulation of aggregated denatured proteins is a common feature in age-dependent neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease and amyotrophic lateral sclerosis (Trojanowski and Lee 2000). In PD, α -synuclein (α SYN) is the major component of Lewy bodies and neurites, the pathological hallmarks of PD (Spillantini et al. 1997), and the filamentous form of α SYN accumulates in degenerating dopamine neurons (Irizarry et al. 1998; Fujiwara et al. 2002). Aggregation and fibril formation of α SYN are now considered to play a role also in the pathogenesis of other α -synucleinopathies, such as dementia with Lewy bodies and multiple system atrophy. In the rare case of early-onset, autosomal dominant forms of PD, the mutations of α SYN gene, A53T (Polymeropoulos et al. 1997), A30P (Kruger et al. 1998) and E46K (Zarranz et al. 2004) and the gene triplication (Singleton et al. 2003) were identified as possible etiologic factors. In transgenic animals expressing wild and mutated human α SYN, a PD-like phenotype was observed, including degeneration of dopamine neurons, formation of α SYN-containing inclusions and the onset of motor dysfunction (Feany and Bender 2000; Masliah et al. 2000).

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These results indicate that α SYN is also involved in the pathogenesis of the sporadic PD through the excess production or the conformational change similar to mutated α SYN (Polymeropoulos et al. 1997; Singleton et al. 2003). However, it remains to be elucidated how α SYN is involved in pathogenic factors, such as increased oxidative stress, mitochondrial dysfunction and impaired ubiquitin-proteasome function to induce the selective cell death in nigral dopamine neurons.

α SYN is a small cytosolic protein of 14 kDa and enriched in presynaptic nerve terminal of the brain, but its physiological function remains unknown. The secondary structure of α SYN depends on the environment. α SYN exists in a random-coil structure in aqueous solution, forms α -helical structure upon binding to phospholipids vesicle and β -sheet structure in soluble fibrils. The amino-terminal region (residues 7–87) of α SYN contains a series of amphipathetic α -helical domains composed of 6 repeats of the amino acid sequence, KTK(E/Q)GV, which is similar to the repeats characteristic for A₂ apolipoproteins, and is associated with lipid membranes (Jo et al. 2000). The central region (residues 61–95) is very hydrophobic and is the same as the fragments isolated from Alzheimer's disease senile plaques. The carboxyl-terminal regions are rich in glutamate and quite acidic. The secondary and primary structure of α SYN account for the interaction with other cellular components.

Point mutation (A30P) of α SYN abolishes the ability to bind to the lipid vesicles (Clayton and George 1999), which will break the equilibrium between membrane-bound and free α SYN and cause the aggregation of free α SYN. Another mutation (A53T) of α SYN does not affect the lipid binding capacity, but reduces synaptosomal membrane fluidity (Jo et al. 2004). Actually, mutated α SYN accelerates the formation of more toxic protofibril (prefibrillar oligomer), but not of fibril (Li et al. 2002). These results suggest that the altered interactions between α SYN and lipids may contribute to the cell death in PD.

In sporadic PD, post-translational events may affect α SYN conformation to increase filamentous and reactive property similar to those of mutated α SYN. In sporadic PD, increased oxidative stress with generation of reactive oxygen and nitrogen species (ROS and RNS), a well-confirmed risk factor, may modify proteins in the nigro-striatal dopamine neurons, as shown by the increased immunoreactivity against protein-bound 4-hydroxy-2-nonenal (HNE), an aldehyde produced by lipid peroxidation, in the nigral neurons of PD brains (Yoritaka et al. 1996). In addition, 3-nitrotyrosine, a product of tyrosine with peroxynitrite, was detected in α SYN in Lewy bodies (Giasson et al.

2000). Considering that α SYN plays a role in lipid transport and synaptic membrane biosynthesis through binding to lipid membrane, it may be reasonable to consider that lipid peroxide and produced aldehydes will conjugate with α SYN proteins and change their conformation. Acrolein (ACR) is another reactive aldehyde produced by lipid peroxidation, and ACR and HNE covalently bind to lysine, histidine and cysteine residues of proteins, or nucleotides by the Michael addition. HNE was reported to bind to α SYN at His⁵⁰ *in vitro* (Trostchansky et al. 2006). However, the occurrence of HNE- and ACR-modified α SYN conjugate in the human brain has never been reported. ACR-protein adduct contains an electrophilic center, which induces severe conformational changes in itself and other proteins through intra- and inter-crosslinking (Furuhata et al. 2002; Burcham et al. 2004).

In this paper, we examined whether α SYN is modified with ACR in the brains of parkinsonian patients using the antibody specific against α SYN and ACR, which was prepared by use of a stable ACR-amino acid adduct, N^ε-(3-formyl-3,4-dehydropiperidino)-lysine (FDP-lysine) as the antigen (anti-ACR antibody) (Uchida et al. 1998). The existence of α SYN-ACR adduct was also studied by the immunoprecipitation of samples prepared from the substantia nigra of control and parkinsonian patients. The aggregation of ACR-modified proteins was examined using human recombinant α SYN, and effects on the proteasome activity was examined using purified proteasome sample and dopaminergic SH-SY5Y cells. The results are discussed in relation to the involvement of α SYN-ACR adduct formation in the production of reactive α SYN oligomer (Lee and Lee 2002) which may induce the cell death of dopamine neurons in PD.

Material and methods

Materials

Autopsied brains from 4 PD patients (age 72 ± 9.8 years, M/F = 2/2) and 4 controls without neurological diseases (age 76 ± 3.4 years, M/F = 1/3) were used for the immunochemical analysis. The substantia nigra from brains of 2 control and 4 parkinsonian patients was isolated by punching out, and stored at -80°C until analysis. The protocol of brain sample analysis was approved by the ethical committee of Aichi Medical University (for formalin-fixed samples) and that of University of Würzburg, (for frozen samples). ATP, lactacystin and ACR were purchased from Sigma-Aldrich (St. Louis, USA); purified 20S proteasome and human recombinant α SYN from BIOMOL International (Butler Pike, PA, USA). 7-Amino-4-methyl-coumarin (AMC) and a fluorescent substrate for proteasome, carboxybenzoxymethyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methyl-coumarin-7-amide (Z-LLVY-MCA) were purchased from Peptide Institute (Osaka, Japan). Anti-ACR monoclonal antibody was purchased from NOF (Tokyo, Japan); polyclonal antibody against C-terminal fragment of α SYN from IBL (Takasaki, Japan) for fluoromicroscopy and from Sigma-Aldrich (St. Louis,

USA) for confocal microscopy. Alexa fluor[®] antibodies were purchased from Molecular Probes (Eugene, OR, USA).

Immunostaining of human brain samples

Paraffin-embedded human midbrain sections containing the substantia nigra were used for immuno-histochemical observation for ACR-adduct protein and α SYN, using DAKO immunostaining kit (DAKO, Kyoto, Japan), as reported (Calingasan et al. 1999). The 8- μ m-thick transverse sections were heated at 60°C for 60 min, deparaffinized and hydrated with graded ethanol solution, then rinsed in 50 mM Tris-HCl, pH 7.5 (Duda et al. 2000). The sample was incubated with the anti- α SYN rabbit polyclonal antibody (IBL) (1:50) and anti-ACR mouse monoclonal antibody (1:100), then with the biotinylated anti-mouse secondary antibody (DAKO, 1:200) for 45 min at the room temperature. The sample was further incubated with Alexa fluor[®] 488 anti-rabbit secondary antibody (1:200) and streptavidin-Alexa Fluor[®] 594 conjugate (1:200) for 45 min. Green fluorescence of Alexa fluor[®] 488 for α SYN and red fluorescence of Alexa fluor[®] 594 for ACR were observed by use of a fluorescence microscope, Olympus BX60 (Olympus, Tokyo, Japan). Nuclei were stained with hematoxylin. Fifty neuromelanin-containing neurons were examined by fluoromicroscopy whether they were stained positively for either α SYN or ACR, or for both of them in the substantia nigra of 4 parkinsonian brains and 4 control brains.

For confocal microscopy observation, the samples were deparaffinized and hydrated, then, incubated with anti- α SYN rabbit polyclonal antibody (Sigma-Aldrich) (1:50) and anti-ACR mouse monoclonal antibody (1:100) for 45 min. The samples were incubated with Alexa-fluore[®] 555 anti-rabbit secondary antibody (1:200) and streptavidine-Alexa fluor[®] 488 conjugated (1:200) for 45 min. Green fluorescence of Alexa fluor[®] 488 for ACR and red fluorescence of Alexa fluor[®] 555 for α SYN were observed by use of the LSM 510 system (Carl Zeiss Microimaging, Jena, Germany).

Immunoprecipitation of ACR-modified α SYN in samples from human brains

Immunoprecipitation of the samples from control and parkinsonian brains was performed, as reported previously (Shamoto-Nagai et al. 2003). In short, 50–100 mg of human brain was lysed in about 8-fold volume of the lysis buffer [10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, Indiana, USA)]. The lysate (2 mg protein) was incubated with 5 μ l of anti- α SYN antibody (primary antibody) (IBL, 10 μ g protein) at 4°C overnight. The mixture was then treated with 25 μ l of protein A-Magnetic beads (New England Biolabs, Ipswich, MA, USA) and incubated at 4°C for 1 h. The mixture was applied to magnetic field and the beads were washed with the lysis buffer for three times. The beads were suspended in the Laemmli sample buffer, boiled for 5 min at 98°C, and the solubilized sample of α SYN and its binding proteins were applied to SDS-PAGE for immunoblotting with anti- α SYN or anti-ACR antibodies. The relative density of the bands positive both α SYN and ACR was quantified by NIH imaging software.

Determination of α SYN modification and aggregation by ACR

α SYN (2.5 μ M in the final concentration) dissolved in 10 mM Tris-HCl buffer, pH 7.5, was incubated in the presence of 0.5, 1, 5, or 10 mM of ACR at 37°C for 20 h. Then, the equal volume of the Laemmli buffer was added to the reaction mixture and the sample was boiled at 100°C for 5 min. The mixture of ACR with α SYN without the incubation was used as a blank. The sample was subjected to Western blot analysis, by SDS-PAGE on 5–20% gradient gel (Wako Pure Chemical Industries, Osaka, Japan), blotted onto PVDF membrane and stained with the anti- α SYN or anti-ACR antibody.

The effect of ACR on 20S proteasome in vitro

The effect of ACR on the activity of 20S proteasome was estimated *in vitro*. The enzyme preparation (50 μ g protein) or purified 20S proteasome (100 μ g, BIOMOL) was incubated with or without 10 mM ACR in the reaction mixture [50 mM Tris-HCl buffer, pH 8.0, containing 1 mM dithiothreitol (DTT) and 0.5 mM EDTA 2Na] for 30 min at 37°C. Then, the substrate Z-LLVY-MCA (50 μ M in the final concentration) was added to the reaction mixture and incubated for another 30 min. The reaction was terminated by adding the same volume of 1% SDS in 100 mM Tris-HCl buffer, pH 8.0. The fluorescence intensity of AMC cleaved by 20S proteasome was quantified at 440 nm with excitation at 380 nm using a Shimadzu spectrofluorometer RF-5300. The activity of the proteasome was expressed as pmol AMC cleaved per min per mg protein (Shamoto-Nagai et al. 2003). The effect of ACR on 20S proteasome derived from SH-SY5Y cells were examined also. The cells were mechanically harvested and washed twice in phosphate-buffered saline (PBS). Then, the cells were homogenized in PBS and centrifuged at 14,000 g for 60 min. Glycerol was added to the supernatant to be 20% in volume, which was used as an enzyme preparation for measurement of 20S proteasome activity. Then, the enzyme preparation was treated with ACR in the same way of the purified 20S proteasome protein.

The production of ACR-adducted proteins was estimated by use of SDS-PAGE followed by immunoblotting as described in the above section.

Effect of ACR-modified α SYN on 20S proteasome activity in vitro

The effect of the α SYN modified by ACR on 20S proteasome was examined further using ACR-modified α SYN. α SYN dissolved in PBS (1 mg/ml) was incubated in the absence or presence of 5 μ M to 5 mM ACR at 37°C for 20 h. Conjugation of ACR to α SYN was confirmed by SDS-PAGE using 5–20% gradient gel and immunoblotting with the antibody against α SYN or ACR. The purified 20S proteasome (100 μ g) was incubated for 30 min in the absence or presence of 10 μ g of native α SYN, or α SYN incubated with 50, 500 μ M, and 5 mM ACR. The activity of chymotrypsin-like 20S proteasome was quantified using Z-LLVY-MCA as a substrate as described above. As a positive control, the effect of 10 μ M lactacystin (Lac), an inhibitor of 20S proteasome, was examined.

Statistics

Experiments were repeated at least 4 times, and the data were expressed as mean \pm SD. Difference was statistically evaluated by analysis of variance (ANOVA), followed by Sheffe's *F*-test. A *p* value less than 0.05 is considered to be statistically significant.

Results

Detection of ACR-modified α SYN in the cytoplasm of neuromelanin-containing dopamine neurons from PD brains

By histopathological observation, α SYN was detected in the dopamine neurons containing neuromelanin in the substantia nigra of PD patients (Fig. 1, A-I and -II). Proteins immunoreactive to anti- α SYN antibody were found in the cytoplasm (Fig. 1, B-I) and mostly co-localized with those stained with anti-ACR antibody. In Table 1, the number of positively immunoreactive neurons was expressed as the percentage of the neuromelanin-containing neurons. In PD, the number of neurons positive for both α SYN and

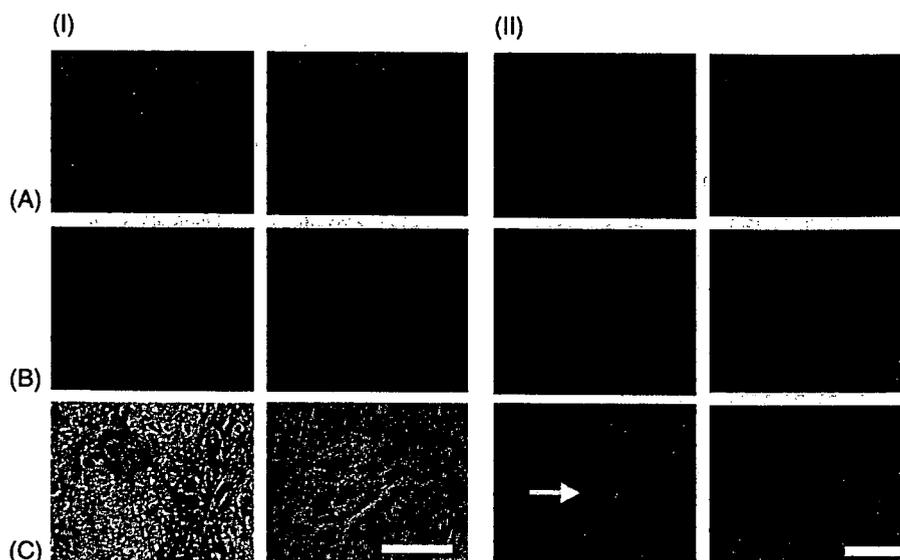


Fig. 1. Co-localization of α SYN and ACR immunoreactivity in the dopamine neurons of the substantia nigra from control and PD patients. Immunoreactivity of α SYN to anti-ACR antibody in dopamine neurons of PD brains. (I) Fluorescence microscopic observation. The immunoreactivity of α SYN (A) was observed in the cytosol and in Lewy body, but that of ACR (B) was not prominent in Lewy bodies. (C) Phase contrast. Bar = 10 μ m. (II) Confocal microscopic observation. The dopaminergic neurons in PD with Lewy bodies, were stained with α SYN (A) and ACR (B). There is co-localization of ACR and α SYN in Lewy body (white arrow, in C). Bar = 10 μ m

Table 1. Incidence of ACR- and α SYN-positive neuromelanin-containing dopamine neurons in the substantia nigra of PD and control patients

	ACR	α SYN	ACR and α SYN
PD ($n=4$)	37.9 \pm 13.7*	47.4 \pm 15.8*	28.4 \pm 26.7*
Control ($n=4$)	8.6 \pm 3.8	13.1 \pm 8.6	3.4 \pm 4.1

Formalin-fixed sections of the human midbrain were stained with antibodies against ACR and α SYN as described in the Material and methods. The percentages of the neuromelanin-containing dopamine neurons immunoreactive to antibody against ACR, α SYN, or both of them are expressed as mean and SD. * $p < 0.05$ by unpaired p -test.

ACR was 28.4% of neuromelanin-containing neurons, whereas in the control brain, only 3.4%. The cells without neuromelanin were neither immunopositive to α SYN or ACR (data, not shown). The microscopic observation was confirmed using another antibody against α SYN and confocal microscopy (Fig. 1, A-II and B-II). The most of the cases, ACR-positive proteins were observed in the cytoplasm but small number of Lewy bodies which are double positive for α SYN and ACR was observed (white arrow). The co-localization of ACR and α SYN was confirmed also under these conditions (Fig. 1, C-II).

Increased formation of ACR-conjugated α SYN was further proved by the immuno-precipitation of α SYN protein. As shown in Fig. 2, in samples from PD patients the protein bands corresponding to α SYN (black arrow) were more intensively stained with the anti-ACR antibody. Fig. 2A shows that anti- α SYN polyclonal antibody used here stained many protein bands, in addition to the band corresponding to α SYN monomer of 14 kDa. In PD brain, intensity of ACR-positive proteins was increased (white arrow), and most of them corresponded with those stained with α SYN antibody (Fig. 2B). Figure 2C shows the quantitative

analyses of the immunoreactivity against ACR in protein band corresponding to α SYN monomer of 14 kDa, as the relative ratio of the density stained with the anti-ACR against that with anti- α SYN antibody. The ACR-modified α SYN significantly increased in the substantia nigra sample from all 4 PD patients.

ACR induced oligomerization and aggregation of α SYN *in vitro*

The effects of ACR on tertiary structure of α SYN were studied *in vitro* using recombinant human α SYN. As shown in Fig. 3, in α SYN samples treated with ACR for 20 h, the band corresponding α SYN monomer with a molecular mass of 14 kDa become broader and reactive to anti-ACR antibody, indicating modification of α SYN by ACR (black arrow). In addition, oligomerized and aggregated α SYN was enhanced in the sample treated with ACR (white arrow). α SYN treated with 10 mM ACR was detected as highly polymerized with random molecular mass, suggesting the destruction and fragmentation of α SYN. The α SYN aggregation was dose-dependent to ACR concentration, and the polymerization and aggregation were not observed in α SYN-ACR mixture before incubation (0 time samples). Figure 3C shows how the ACR modification induces cross-reaction of protein and polymerization.

ACR reduced the activity of 20S proteasome *in vitro*

Effect of ACR on the activity and high structure of 20S proteasome in the cytoplasmic fraction of the purified sample and SH-SY5Y cells were examined *in vitro*. ACR markedly reduced the activity of 20S proteasome in both

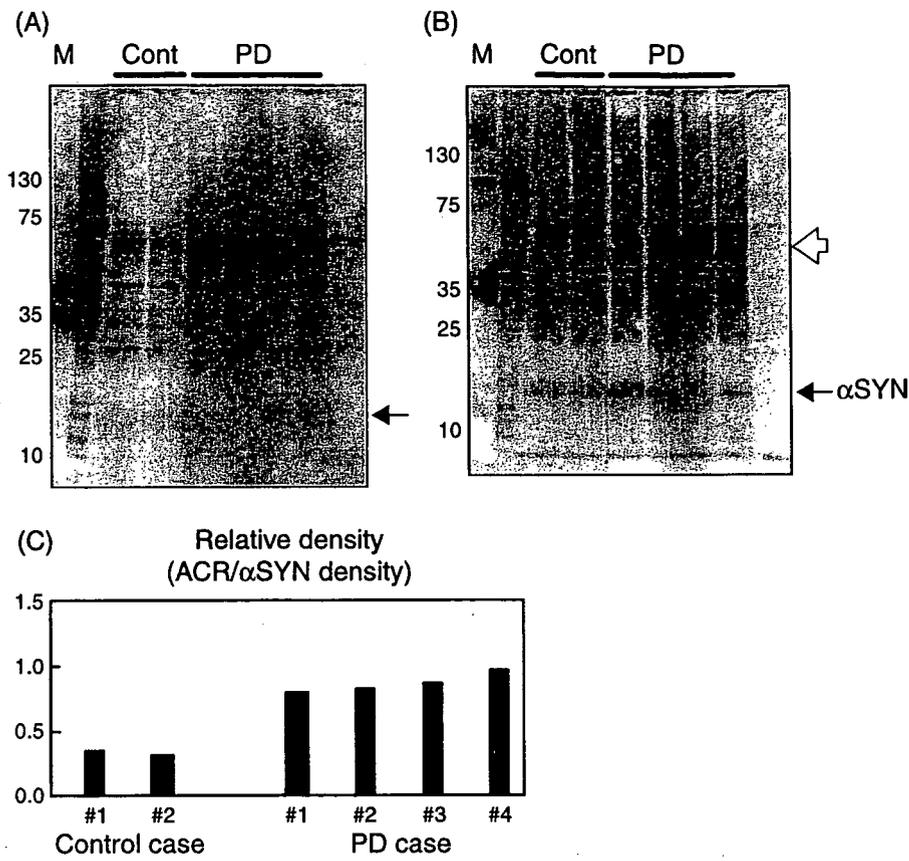


Fig. 2. Modification of α SYN by ACR in the PD brains. Samples from the substantia nigra of control (*Cont*) and PD patients (*PD*) were homogenized and immunoprecipitated with antibody against α SYN. The samples were subjected to SDS-PAGE and detected using anti- α SYN (A) and anti-ACR antibody (B) as described in the Material and methods. (C) The relative density of immuno-staining of the protein band stained with anti-ACR antibody against that with anti- α SYN antibody was assessed using the image analysis software. α SYN in samples prepared from PD brains was more markedly stained against anti-ACR antibody (*black arrow*). In PD brain samples, the increase of ACR-modified protein was observed (*white arrow*) (B)

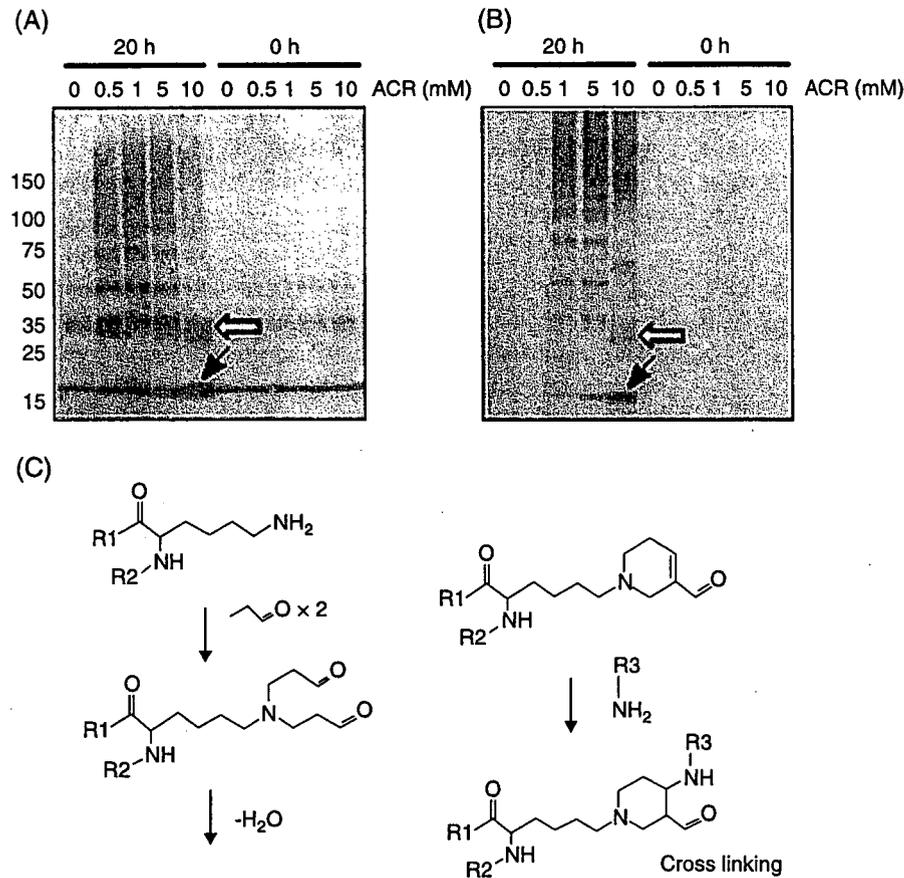


Fig. 3. Oligomerization and aggregation of α SYN induced by ACR-modification. α SYN ($2.5 \mu\text{M}$) was incubated in the presence of 0.5, 1, 5, or 10 mM of ACR at 37°C for 20 h as described in the Material and methods. Then, the samples were separated by SDS-PAGE and blotted using anti- α SYN or anti-ACR antibody. (A) Immunoblotting using anti- α SYN antibody. (B) Immunoblotting using anti-ACR antibody. *Black arrow* α SYN monomer was modified by ACR. *White arrow* in the sample incubated with ACR, oligomerization of α SYN was detected. (C) The scheme of chain reaction of ACR with amino acids to produce cross-linking of proteins

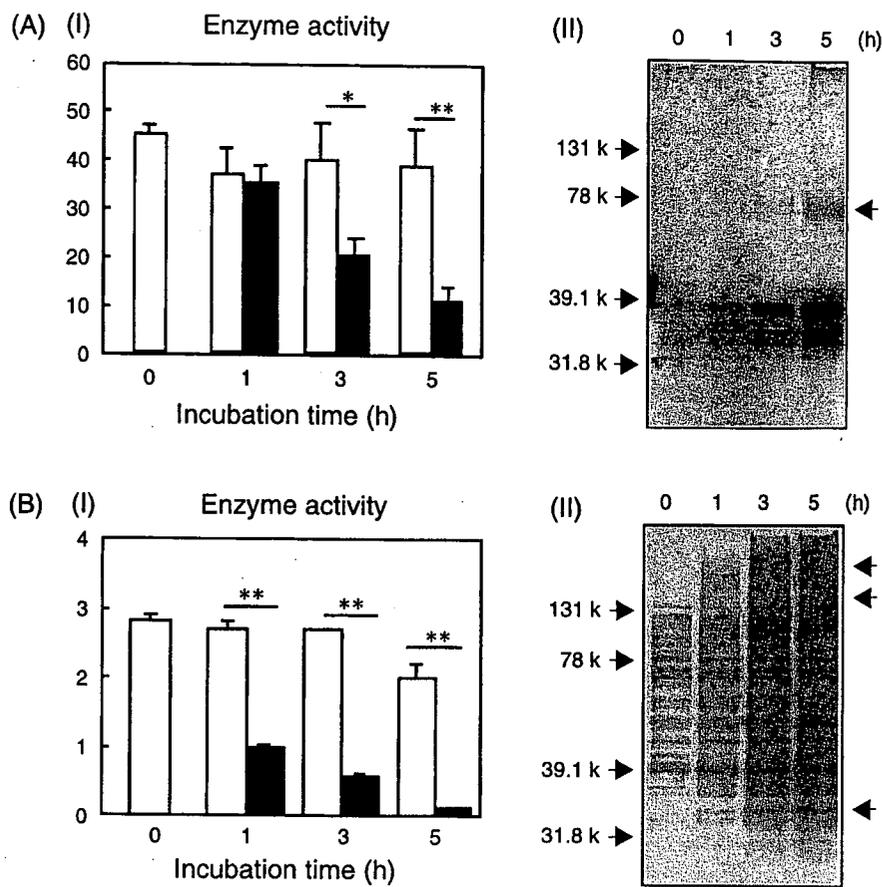


Fig. 4. Modification of 20S proteasome by ACR and reduction of the enzyme activity *in vitro*. The enzyme sample prepared from the purified 20S proteasome (A) or cytoplasmic fraction of SH-SY5Y cells (B) was incubated without or with 1 mM of ACR solution for 1 to 5 h. (A-I) and (B-I): Chymotrypsin-like activity of 20S proteasome was measured fluorometrically using LLVY-AMC as a substrate, as described in Material and methods. The column and bar represent the mean and SD of 4 independent experiments. Difference from the activity in control treated without ACR was significant (** $p < 0.01$) by ANOVA. (A-II) and (B-II): The samples are separated by SDS-PAGE and visualized with antibody against ACR. After incubation of the cytosol with ACR, increased number of the proteins immunoreactive to anti-ACR antibody was observed, indicating that ACR adducted multiple proteins [(B-II)]. Adduct formation of ACR with 20S proteasome was identified by use of purified enzyme sample [(A-II)]

the samples (Fig. 4, A-I and B-I). The inhibition of the activity was more potent in the cytoplasmic enzyme preparation than in the purified enzyme. The activity of 20S proteasome was virtually undetectable in the cytoplasmic enzyme preparation after 5 h incubation with 10 mM ACR. By Western blot analysis of the cytoplasmic sample, numerous proteins were modified with ACR (Fig. 4, B-II). The purified 20S proteasome were also modified by ACR with aggregation to high polymers (Fig. 4, A-II).

α SYN conjugated with ACR inhibited 20S proteasome activity in vitro

The direct effect of ACR-modified α SYN on the 20S proteasome activity was examined *in vitro*. After the incubation with ACR, polymerized α SYN conjugated with ACR (ACR-SYN) increased in a dose-dependent way to ACR (Fig. 5A). In the α SYN sample incubated with 5–100 μ M ACR, the dimmer, tetramer and higher polymers of α SYN were observed as ladder formation, whereas with 500 μ M and 5 mM ACR, α SYN was cleaved and aggregated showing smear pattern (Fig. 5A). As shown in Fig. 5C, α SYN treated with 5 mM of ACR for 20 h inhibited 20S protea-

some activity significantly ($p < 0.001$). Pre-treatment of 100 μ g of enzyme sample with 10 μ g ACR-SYN adduct reduced the activity to be $40.0 \pm 4.5\%$ of control. On the other hand, native α SYN did not affect the 20S proteasome activity at all.

Discussion

Our results show that in the substantia nigra from PD patients ACR-modified α SYN accumulates mainly in the cytoplasm of the nigral melanized neurons. Western blot analyses of the lysate immuno-precipitated with anti- α SYN antibody confirmed the increased ACR-modification of α SYN in the substantia nigra of parkinsonian brains. α SYN localizes on the lipid bilayer of the synaptic vesicles and lipid rafts of the presynaptic terminal (Kahle et al. 2000; Zhu et al. 2003; Fortin et al. 2004; Nuscher et al. 2004), and it exists in three conformations: lipid bound α -helices, unfolded in solution and fibrils (Kessler et al. 2003). The protein structure of α SYN contains seven imperfect repeats of 11 amino acids, forming N-terminal α -helices, a central hydrophobic domain and acidic C-terminal rich in glutamate. The primary structures and con-

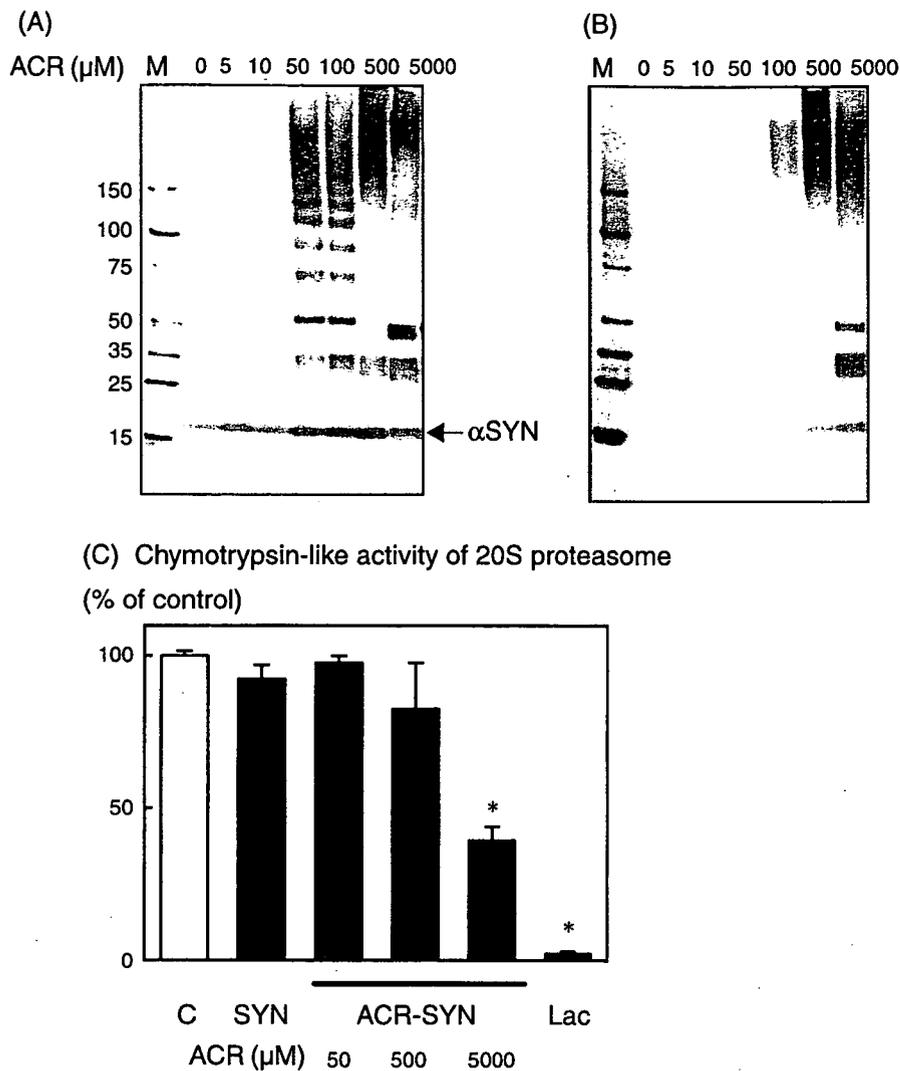


Fig. 5. Inhibition of purified 20S proteasome activity by ACR-modified α SYN (ACR-SYN). α SYN was incubated without or with 5 μ M to 50 mM ACR for 20 h and the sample was subjected to SDS-PAGE and detected with anti- α SYN antibody (A) or anti-ACR antibody (B) as described in the Material and methods. α SYN (arrow) was conjugated with ACR in a dose-dependent manner. (C) The 20S proteasome sample was pre-incubated in the absence or presence of α SYN or α SYN modified by ACR (ACR-SYN) for 30 min. Then, the chymotrypsin-like activity of 20S proteasome was measured using a synthesized substrate, LLVY-AMC. C The enzyme sample without α SYN, SYN enzyme sample incubated with native α SYN, ACR-SYN enzyme sample incubated with ACR-treated α SYN at the indicated concentrations. * $p < 0.001$ compared to control by ANOVA

formation suggest that α SYN may function in protein-membrane, namely protein-lipid, interaction. The interaction of α SYN with lipids has been well confirmed and the association with lipids induced the oligomer formation (Eliezer et al. 2001). The dissociation of α SYN from lipids may transform the primarily random-coil secondary structure to β -sheet rich structure, which promotes protofibril formation in cytoplasm. α SYN oligomer and protofibril are now proposed to be cytotoxic by permeabilization of membranes (Volles et al. 2001; Rochet et al. 2004), increased generation of ROS and RNS (Xu et al. 2002), and elevated levels of α SYN β -sheet (Petrucci et al. 2002). These results suggest that factors regulating the α SYN conformation, the affinity to lipid layer and the equilibrium between the monomer, oligomer, protofibril and fibril form, may play an important role in the formation of the inclusion body, and the cell death of nigral dopamine neurons.

In addition to α SYN gene mutation, the oxidative modification will induce the conformational changes in α SYN, and its close localization to lipid bilayer suggests the products of lipid peroxidation, aldehydes and radicals, may mediate the oxidative modification. ACR modifies protein by adduct formation with the imidazole group of histidine, amino group of lysine, and sulfhydryl group of cysteine, then ACR undergoes nucleophilic addition at the double bond to form a secondary derivative with the retention of the aldehyde group, resulting in the formation of the Michaelis addition-type ACR-amino acid adducts. ACR modification of histidine and lysine produce further 3-(*N*-imidazole)propanol and *N*^ε-(3-formyl-3,4-dehydropiperidino)-lysine (FDP-lysine), respectively. FDP-lysine reacts with sulfhydryl groups to form thioether adducts (Furuhata et al. 2002). This reaction may accelerate the cross linkage of between α SYN and other proteins (Fig. 3C). Even

though the half life of free ACR was very short by *in vitro* and *ex vivo* experiments, ACR modifies protein very effectively in a short time (Uchida et al. 1998). Indeed, incubation of α SYN with ACR rapidly produced aggregated ACR-modified protein (Figs. 3 and 5). Figure 5 shows that ACR at 50 μ M already induces α SYN polymerization. At present the exact concentration of ACR in human brain has not been determined, but in rat tissues the HNE concentration was estimated around 3 μ M, which increased to 10 μ M by oxidative stress (Esterbauer et al. 1990). Considering dysfunction of the UPS in PD, it may be reasonable to consider that ACR even lower concentrations may modify α SYN, resulting in the accumulation in dopamine neurons of aged and parkinsonian brain. In addition, ACR itself inhibits the activity of proteasome, as discussed below.

In oligomerized α SYN, the immunoreactivity against ACR was not prominent compared to α SYN monomer. It may be ascribed to that ACR binding site and/or the recognition site of anti-ACR antibody were not fully any more exposed in aggregated α SYN as in the case of Lewy body. This may explain also the different distribution of ACR- and α SYN-positive cells in neuromelanin-containing neurons, and why only a third of neurons were stained with both the antibodies (Table 1). In addition, conformational changes of ACR modified α SYN into the oligomer, filamentous or insoluble form may explain the limited increase in ACR- α SYN adducts in immuno-precipitated sample from PD patients (Fig. 2).

The possible toxicity of ACR and ACR modified α SYN was studied especially in concern with proteasome activity. Impairment of the proteolysis system has been gathering attention as a mechanism of neuronal cell death in PD. Gene mutation and inactivation of the enzymes of the UPS, including parkin, E3 ubiquitin-ligase (Kitada et al. 1998) and ubiquitin C-terminal hydrolase L1 (Leroy et al. 1998), were identified as pathogenic factors in autosomal recessive familial PD. Also in sporadic cases of PD, reduced activity of 20S proteasome was reported in the striatum (McNaught and Jenner 2001). As shown in Fig. 4, B-I, free ACR markedly reduced 20S proteasome activity in the cytoplasmic enzyme preparation, and the mechanism behind the inhibition of the activity was studied. ACR was found to adduct with many components of 20S proteasome proteins as shown in the purified enzyme proteins incubated with ACR (Fig. 4, A-II). ACR may directly modify the proteasomal proteins, then, induce conformational change with inactivation of the enzyme. It was further demonstrated using purified 20S proteasome, the activity of which was reduced, according to the modification and aggregation of proteasomal protein (Fig. 4, B-II).

Another mechanism of the inhibition is the effect of ACR-adduct proteins on proteasome. Oxidative-modified protein is a substrate and inhibitor of 20S proteasome system (Shringarpure et al. 2003). We found that rotenone, a complex I inhibitor, induced the reduction of 20S proteasome activity and the accumulation of aggregated ACR-modified proteins to induce apoptotic cell death in human neuroblastoma SH-SY5Y cells (Shamoto-Nagai et al. 2003). In that system ACR-modified protein was co-immunoprecipitated with 20S β subunit, the active site of 20S proteasome. In this article ACR-modified proteins was shown to inhibit 20S proteasome activity directly. The relatively weak potency of inhibition by ACR-SYN may be ascribed to that the molecular size of α SYN protein is much larger than that of the synthesized peptide substrate. Proteasome system is composed of a cylinder-like structure and in the hole disentangled proteins are cleaved by enzymes into small peptides. The peptides or protein fragments released from ACR-modified α SYN or other proteins may act as an endogenous inhibitor of the proteasome system.

The modification of α SYN with ACR may initiate the accumulation of abnormal proteins by impairment of the UPS. A vicious cycle of oxidative stress, mitochondrial dysfunction and reduced UPS activity may culminate in neuronal cell death with accumulation of oligomeric α SYN in the sporadic form of PD.

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いるので、DHA が健康の維持に重要だからといって、単独を多量に摂取すると、かえって PUFA 全体の代謝を乱すことになる。

■ PUFA 摂取には抗酸化物質の共摂取が必須

PUFA の二重結合は活性酸素の攻撃を受け、ペルオキシラジカルを形成しやすい。これを防ぐために、ビタミン C, E, β -カロテン、ポリフェノールなどの抗酸化剤と一緒に摂取することが必要である¹⁴⁾。

■ PUFA の推薦摂取量

脂肪摂取総量は全エネルギー摂取量の 20% ~25% の範囲に収め、PUFA の摂取総量は全エネルギーの 10% にするのがよいとされている。

■ おわりに

最近メタボリックシンドロームの防止のために脂肪の摂食を避ける傾向にある。しかし、n-3系と n-6系の PUFA は健康の維持に欠かすことのできない重要な機能をもつうえに、栄養学うえ必ず摂取しなければならない必須脂肪酸 (EFF)^{*1}である。

ただし、n-6系の PUFA は過剰になると炎症、血小板凝集などにみられるように病的現象の発現と密接な関係をもつことから、重要性が見逃されている傾向がある。n-6系と n-3系 PUFA は同じ酵素群によって代謝されるため、一方の量が多いと、他方の欠乏が起こる。そのことが、たとえば DHA と AA を単純に比較し

た場合に AA の負の面が浮き彫りにされる。今後は、異なる n-6/n-3をもつ PUFA を含む食事を与えて、AA など n-6系の PUFA の機能を見直す必要があると思われる。

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*1: 上述のように、厳密な意味では ALA と LA が EFF に当たる。また、エイコサノイド類の発見に至るまでの経緯から AA を加えて 3 種の PUFA が EFF とされてきたこともある。しかし、最近では Δ^6 不飽和酵素の活性が制限されているため、たとえば、n-3系の PUFA を供給するのに ALA よりむしろ EPA や DHA を与えると効率がよいという意味で、n-3系、n-6系の PUFA のすべてを EFF とするのが趨勢である。

野菜(植物性食品)摂取の効果

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keyword

ビタミン, ファイトケミカル, 抗酸化機能,
遺伝子発現制御

はじめに

野菜(植物性食品)は食品の重要な要素であるとともに、必須栄養素を含む。それに加え、野菜に含まれる種々の成分が脳の老化、老年病を防御する可能性について注目されている。近年、野菜の摂取が脳の老化を防ぎ、老化にともなう神経変性疾患であるアルツハイマー病やパーキンソン病の発症率を低下させることが疫学的に報告された。

本稿では野菜と脳の老化をめぐる最近のトピックを中心に概説する。もちろん、これらのすべてが証明されたわけではなく、今後の研究が待たれる。

野菜に含まれる微量機能性成分

栄養素(生体の維持に必要な化学物質で食物から摂取されるもの、たんぱく質、脂質、炭水化物の三大栄養素にビタミン、ミネラルを加えたものを五大栄養素という)のなかでもビタミンは、野菜から摂取されるものが大部分である。そのなかでもビタミンCやビタミンEのように直接的に抗酸化作用を有することや、葉酸、ビタミンB₁₂、B₆のように認知症のリスクファクターであるホモシステインの代謝を行うこと

で、認知症発症を抑制することが期待されているビタミンも多い。

野菜にはいわゆる栄養素や繊維のほかに種々の色素、スパイスなどの微量成分が含まれる。これらの微量成分はその欠乏によっても欠乏症をきたすことがなく、生体の維持に必須ではないためビタミンではない。しかしこれらの一部は薬理作用をもち、薬品、民間薬として使用されている。それだけでなくこれらの日常摂取により疾病の発症を抑制することが期待され、その成分の多くはファイト(=ギリシャ語で植物)ケミカル(化学物質)と呼ばれる(表1)。

認知症モデルに対する野菜由来成分の効果

ヒト認知症の代表例としてアルツハイマー病がある。高齢化が進むわが国においては年々その患者数が増加しており、65歳以上の高齢者の5%が本疾患に罹患するとされる。アルツハイマー病の真の病因は不明であるが、脳内にベータアミロイド(A β)と呼ばれる構造異常蛋白質が凝集し、蓄積することが神経細胞死の直接の引き金となっているとの仮説が広く受け入れられている(アミロイド仮説)。事実、A β の前駆物質である amyloid precursor protein (APP)の変異により、アルツハイマー病と同様な病理変化をもたらされることは本仮説を支持するものである(図1)。

APP 遺伝子にヒトと同様な変異を起こした

表1 ファイトケミカルの代表例とそれを含む植物性食品

ポリフェノール	イソフラボン類	大豆
	アントシアニン	ブルーベリー
	レスベラトロール	赤ワイン
	カテキン類	緑茶
	クルクミン	ウコン
	フラバノン類	柑橘類
	リグナン類	ごま
有機硫黄化合物	スルフォラファン	ブロッコリー, キャベツ
	アリシン	にんにく
テルペノイド	ルテイン	ほうれんそう
	リコペン	トマト
	beta-クリプトキサンチン	柑橘類
	カプサイシン	とうがらし
糖関連化合物	beta-グルカン	きのこ
	ペクチン	りんご

遺伝子改変マウスの脳には $A\beta$ の蓄積が認められる。近年, $A\beta$ に対する免疫を賦活することで脳内の $A\beta$ 凝集体(老人斑)が減少することが報告され, 注目を集めている¹⁾。現在, 世界各国でヒトに対する $A\beta$ 免疫療法が試みられているが, 副作用(脳炎など)の問題もあり, いまだ実用化はなされていない。一方, 野菜由来の食品成分のなかでもポリフェノールであるクルクミン²⁾やレスベラトロール³⁾, カテキン⁴⁾などがAPP 遺伝子改変マウスの脳内の老人斑を減少させたり, $A\beta$ の繊維化を抑制する⁵⁾との知見が得られている。これら食品由来成分は $A\beta$ の合成を抑制したり, あるいは $A\beta$ の凝集を抑制することで脳内 $A\beta$ 蓄積を減少させたと考えられるが, 詳細なメカニズムは不明である。また, ヒトにおいても同様な効果が得られるかどうかはわかっていない。

ヒト認知症に対する野菜成分の効果

ヒト認知症に対する野菜成分の効果を検証するアプローチとしては2つの方法論がある。疫学的研究と, 介入研究である。前者はいわゆる観察研究であり, 認知症を発症した群と発症していない群を比較し, 生活習慣, 遺伝的背景な

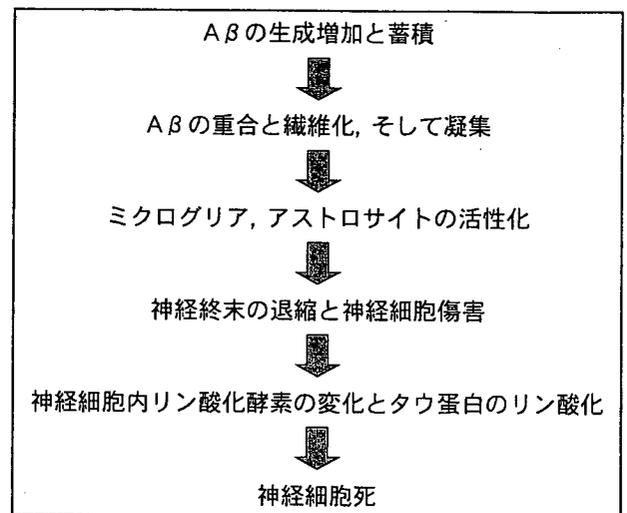


図1 アルツハイマー病のアミロイド仮説
最初になんらかの原因(遺伝子変異, 老化, 酸化ストレスなど)でベータアミロイド($A\beta$)の増加と蓄積が起こり, 細胞死のカスケードが起動される。

どに有意差があるか検定する横断研究, 多数例からなるコホートを追跡し, そのなかで認知症を発症した群とそうでない群を比較する縦断研究がある。横断研究は結果が迅速に得られるという利点があるが, 世代間の差(生活習慣の変化など)のバイアスがかかりやすいという問題点がある。縦断研究はより正確なデータが得られるものの, 解析のためのデータを得るためには膨大な時間と手間がかかるという難点がある。

一方, 介入研究は基本的に薬剤開発で用いられる手法と同様な方法論が使用されることが多