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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 (αSYN) plays a central role in the neural degeneration of Parkinson's disease (PD) through its conformational change. In PD, αSYN, released from the membrane, accumulates in the cytoplasm and forms Lewy body. However, the mechanism behind the translocation and conformational change of αSYN 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, αSYN was modified with acrolein (ACR), an aldehyde product of lipid peroxidation. Histopathological observation confirmed the co-localization of protein immunoreactive to anti-αSYN and ACR antibody. By Western blot analyses of samples precipitated with either anti-αSYN or anti-ACR antibody, increase in ACR-modified αSYN was confirmed in PD brain. Modification of recombinant αSYN by ACR enhanced its oligomerization, and at higher ACR concentrations αSYN 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 αSYN, 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
αSYN α-synuclein
HNE 4-hydroxy-2-nonenal
PD Parkinson's disease
RNS reactive nitrogen species
ROS reactive oxygen species
UPS ubiquitin-proteasome system

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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 (αSYN) is the major component of Lewy bodies and neurites, the pathological hallmarks of PD (Spillantini et al. 1997), and the filamentous form of αSYN accumulates in degenerating dopamine neurons (Irizarry et al. 1998; Fujiwara et al. 2002). Aggregation and fibril formation of αSYN 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 αSYN 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 αSYN, a PD-like phenotype was observed, including degeneration of dopamine neurons, formation of αSYN-containing inclusions and the onset of motor dysfunction (Feany and Bender 2000; Masliah et al. 2000).

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

αSYN 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 αSYN depends on the environment. αSYN 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 αSYN 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 A_2 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 αSYN account for the interaction with other cellular components.

Point mutation (A30P) of αSYN abolishes the ability to bind to the lipid vesicles (Clayton and George 1999), which will break the equilibrium between membrane-bound and free αSYN and cause the aggregation of free αSYN. Another mutation (A53T) of αSYN does not affect the lipid binding capacity, but reduces synaptosomal membrane fluidity (Jo et al. 2004). Actually, mutated αSYN 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 αSYN and lipids may contribute to the cell death in PD.

In sporadic PD, post-translational events may affect αSYN conformation to increase filamentous and reactive property similar to those of mutated αSYN. 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 αSYN in Lewy bodies (Giasson et al. 2000). Considering that αSYN 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 αSYN 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 αSYN at His^{50} *in vitro* (Trostchansky et al. 2006). However, the occurrence of HNE- and ACR-modified αSYN 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 αSYN is modified with ACR in the brains of parkinsonian patients using the antibody specific against αSYN 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 αSYN-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 αSYN, 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 αSYN-ACR adduct formation in the production of reactive αSYN 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 $-80^{\circ}C$ 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 αSYN from BIOMOL International (Butler Pike, PA, USA). 7-Amino-4-methyl-coumarin (AMC) and a fluorescent substrate for proteasome, carbobenzoxy-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methyl-coumaryl-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 αSYN 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 αSYN, using DAKO immunostaining kit (DAKO, Kyoto, Japan), as reported (Calingasan et al. 1999). The 8-μm-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-αSYN 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 αSYN and red fluorescence of Alexa flour® 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 αSYN 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-αSYN 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 fluoresscence of Alexa fluor® 488 for ACR and red fluorescent of Alexa fluor® 555 for αSYN were observed by use of the LSM 510 system (Carl Zeiss Microinaging, Jena, Germany).

Immunoprecipitation of ACR-modified αSYN 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 lyzed 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 μl of anti-αSYN antibody (primary antibody) (IBL, 10 μg protein) at 4°C overnight. The mixture was then treated with 25 μl 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 αSYN and its binding proteins were applied to SDS-PAGE for immunoblotting with anti-αSYN or anti-ACR antibodies. The relative density of the bands positive both αSYN and ACR was quantified by NIH imaging software.

Determination of αSYN modification and aggregation by ACR

αSYN (2.5 μM 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 αSYN 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-αSYN 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 μg protein) or purified 20S proteasome (100 μg, 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 μM 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 αSYN on 20S proteasome activity in vitro

The effect of the αSYN modified by ACR on 20S proteasome was examined further using ACR-modified αSYN. αSYN dissolved in PBS (1 mg/ml) was incubated in the absence or presence of 5 μM to 5 mM ACR at 37°C for 20 h. Conjugation of ACR to αSYN was confirmed by SDS-PAGE using 5–20% gradient gel and immunoblotting with the antibody against αSYN or ACR. The purified 20S proteasome (100 μg) was incubated for 30 min in the absence or presence of 10 μg of native αSYN, or αSYN incubated with 50, 500 μM, 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 μM lactacystin (Lac), an inhibitor of 20S proteasome, was examined.

Statistics

Experiments were repeated at least 4 times, and the data were expressed as mean ± 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 αSYN in the cytoplasm of neuromelanin-containing dopamine neurons from PD brains

By histopathological observation, αSYN 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-αSYN 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 αSYN and

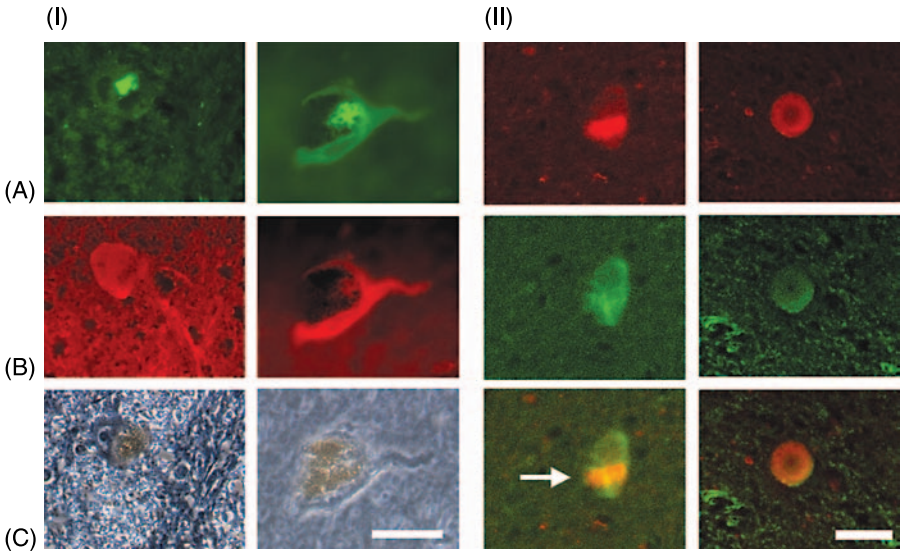

Fig. 1. Co-localization of αSYN and ACR immunoreactivity in the dopamine neurons of the substantia nigra from control and PD patients. Immunoreactivity of αSYN to anti-ACR antibody in dopamine neurons of PD brains. (I) Fluorescence microscopic obsevation The immunoreactivity of αSYN (**A**) was observed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

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

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

Formalin-fixed sections of the human midbrain were stained with antibodies against ACR and αSYN as described in the Material and methods. The percentages of the neuromelanin-containing dopamine neurons immunoreactive to antibody against ACR, αSYN, 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 αSYN or ACR (data, not shown). The microscopic observation was confirmed using another antibody against αSYN 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 αSyn and ACR was observed (white arrow). The co-localization of ACR and αSYN was confirmed also under these conditions (Fig.1, C-II).

Increased formation of ACR-conjugated αSYN was further proved by the immuno-precipitation of αSYN protein. As shown in Fig. 2, in samples from PD patients the protein bands corresponding to αSYN (black arrow) were more intensively stained with the anti-ACR antibody. Fig. 2A shows that anti-αSYN polyclonal antibody used here stained many protein bands, in addition to the band corresponding to αSYN monomer of 14 kDa. In PD brain, intensitiy of ACR-positive proteins was increased (white arrow), and most of them corresponded with those stained with αSYN antibody (Fig. 2B). Figure 2C shows the quantitative analyses of the immunoreactivity against ACR in protein band corresponding to αSYN monomer of 14 kDa, as the relative ratio of the density stained with the anti-ACR against that with anti-αSYN antibody. The ACR-modified αSYN significantly increased in the substantia nigra sample from all 4 PD patients.

ACR induced oligomerization and aggregation of αSYN in vitro

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

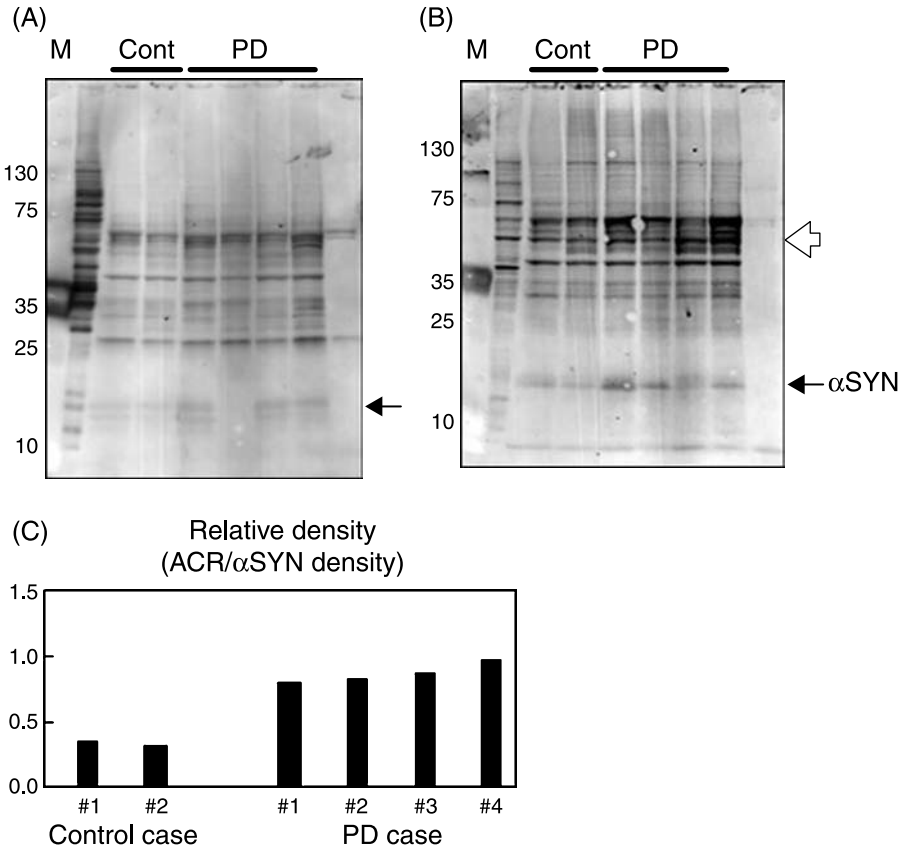

Fig. 2. Modification of αSYN by ACR in the PD brains. Samples from the substantia nigra of control (*Cont*) and PD patients (*PD*) were homogenized and immunoprecipitated with antibody against αSYN. The samples were subjected to SDS-PAGE and detected using anti-αSYN (**A**) and anti-ACR antibody (**B**) 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-αSYN antibody was assessed using the image analysis software. αSYN 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**)

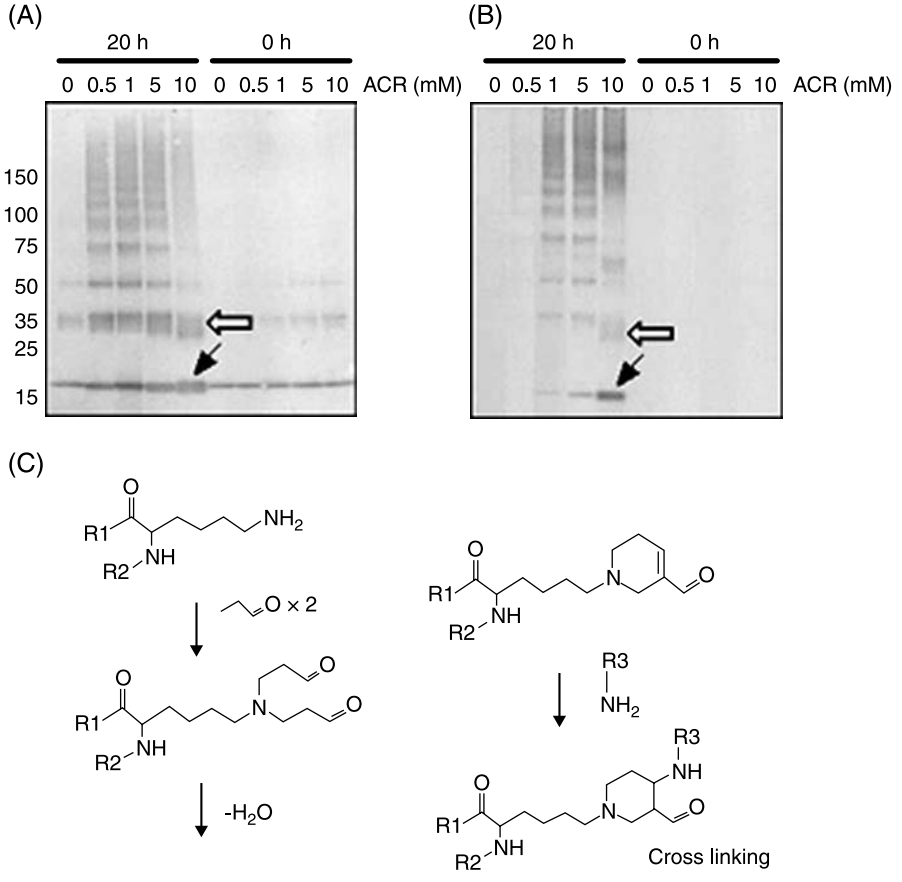

Fig. 3. Oligomerization and aggregation of αSYN induced by ACR-modification. αSYN (2.5 μM) was incubated in the presence of 0.5, 1, 5, or 10 mM of ACR at 37°C for 20 h as described in the Material and methods. Then, the samples were separated by SDS-PAGE and blotted using anti-αSYN or anti-ACR antibody.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

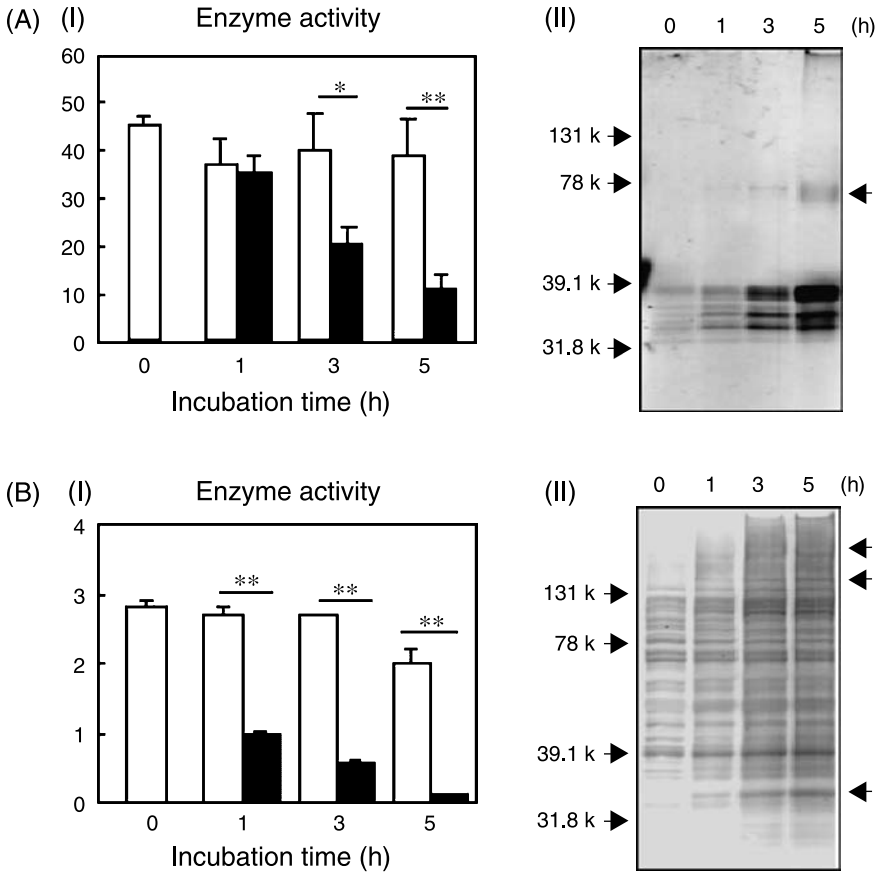

Fig. 4. Modification of 20S proteasome by ACR and reduction of the enzyme activity *in vitro*. The enzyme sample prepared from the purified 20S proteasome (**A**) or cytoplasmic fraction of SH-SY5Y cells (**B**) was incubated without or with 1 mM of ACR solution for 1 to 5 h. (A-I) and (B-I): Chymotrypsin-like 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).

αSYN conjugated with ACR inhibited 20S proteasome activity in vitro

The direct effect of ACR-modified αSYN on the 20S proteasome activity was examined *in vitro*. After the incubation with ACR, polymerized αSYN conjugated with ACR (ACR-SYN) increased in a dose-dependent way to ACR (Fig. 5A). In the αSYN sample incubated with 5–100 μM ACR, the dimmer, tetramer and higher polymers of aSYN were observed as ladder formation, whereas with 500 μM and 5 mM ACR, αSYN was cleaved and aggregated showing smear pattern (Fig. 5A). As shown in Fig. 5C, αSYN treated with 5 mM of ACR for 20 h inhibited 20S proteasome activity significantly ($p < 0.001$). Pre-treatment of 100 μg of enzyme sample with 10 μg ACR-SYN adduct reduced the activity to be $40.0 \pm 4.5\%$ of control. On the other hand, native αSYN did not affect the 20S proteasome activity at all.

Discussion

Our results show that in the substantia nigra from PD patients ACR-modified αSYN accumulates mainly in the cytoplasm of the nigral melanized neurons. Western blot analyses of the lysate immuno-precipitated with anti-αSYN antibody confirmed the increased ACR-modification of αSYN in the substantia nigra of parkinsonian brains. αSYN 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 αSYN 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-

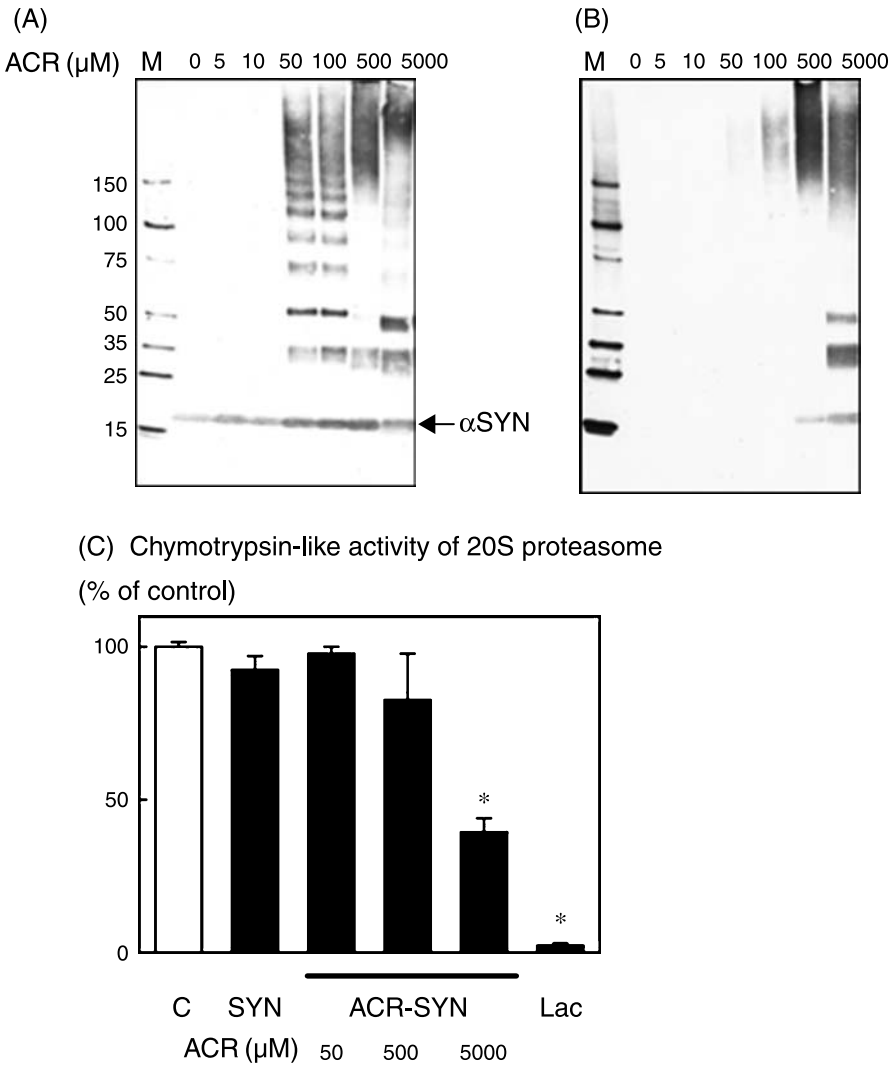

Fig. 5. Inhibition of purified 20S proteasome activity by ACR-modified αSYN (*ACR-SYN*). αSYN was incubated without or with 5 μM to 50 mM ACR for 20 h and the sample was subjected to SDS-PAGE and detected with anti-αSYN antibody (**A**) or anti-ACR antibody (**B**) as described in the Material and methods. αSYN (*arrow*) was conjugated with ACR in a dose-dependent manner. (**C**) The 20S proteasome sample was pre-incubated in the absence or presence of αSYN or αSYN 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 αSYN, *SYN* enzyme sample incubated with native αSYN, *ACR-SYN* enzyme sample incubated with ACR-treated αSYN at the indicated concentrations. $^{*}p < 0.001$ compared to control by ANOVA

formation suggest that αSYN may function in protein-membrane, namely protein–lipid, interaction. The interaction of αSYN with lipids has been well confirmed and the association with lipids induced the oligomer formation (Eliezer et al. 2001). The dissociation of αSYN from lipids may transform the primarily random-coil secondary structure to β-sheet rich structure, which promotes protofibril formation in cytoplasm. αSYN 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 αSYN β-sheet (Petrucelli et al. 2002). These results suggest that factors regulating the αSYN 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 αSYN gene mutation, the oxidative modification will induce the conformational changes in αSYN, 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 sulfhydril groups to form thioether adducts (Furuhata et al. 2002). This reaction may accelerate the cross linkage of between αSYN 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 αSYN with ACR rapidly produced aggregated ACR-modified protein (Figs. 3 and 5). Figure 5 shows that ACR at 50 μM already induces αSYN 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 μM, which increased to 10 μM 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 αSYN, 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 αSYN, the immunoreactivity against ACR was not prominent compared to αSYN 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 αSYN as in the case of Lewy body. This may explain also the different distribution of ACR- and αSYN-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 αSYN into the oligomer, filamentous or insoluble form may explain the limited increase in ACR-αSYN adducts in immuno-precipitated sample from PD patients (Fig. 2).

The possible toxicity of ACR and ACR modified αSYN 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 αSYN 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 αSYN or other proteins may act as an endogenous inhibitor of the proteasome system.

The modification of αSYN 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 oligometric αSYN in the sporadic form of PD.

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