and GLD, that were derived from DHA and AA, respectively (Fig. 1*A*). The chemical structures of the authentic adducts were identified by NMR (supplemental Figs. S1–S4). The formation of these dopamine adducts was further confirmed by HPLC-MS/MS analysis. Collision-induced dissociation of the authentic adducts SUD (*m/z* 254), PRD (*m/z* 210), HED (*m/z* 252), and GLD (*m/z* 268) produced the same daughter ions at *m/z* 91 and 137. SUD, PRD, and HED also produced daughter ions at *m/z* 154, whereas GLD did not. These ions were assigned the structures shown in Fig. 1*B*. The ion at *m/z* 137 was detected with the highest peak intensity in the fragments, and this ion was also identified to be derived from the dopamine spectra.

In Vitro Detection of Dopamine Adducts—To determinate the *in vitro* formation of the dopamine adducts, the reaction of dopamine with DHA or AA hydroperoxides were carried out. The reaction mixture was analyzed by HPLC-MS/MS based on the information in the collision-induced dissociation spectra. As shown in Fig. 2, the peaks indicating SUD, PRD, HED, and GLD were successfully detected at *m/z* 254 → 137, *m/z* 210 → 137, *m/z* 252 → 137, and *m/z* 268 → 137, respectively. The retention times were consistent with those of the authentic adducts.

In Vivo Detection of Dopamine Adducts—It has been reported that polyunsaturated fatty acids such as DHA and AA are significantly enriched in the brain (20) and that there are high levels of basal oxidative stress in the normal brain, which increases with aging (21). To investigate whether the DHA- and AA-derived dopamine adducts can be formed *in vivo*, the brains of 7- and 27-week-old male F344/NSIc rats were removed, and the homogenates were used. The detection of the dopamine adducts in the homogenates was carried out by HPLC-MS/MS. The whole adducts were detected in the 7- and 27-week rat brains in both the positive ion mode and negative ion mode of liquid chromatography-MS/MS (data not shown). The level of adduct formation was shown in Fig. 3. The HED and PRD, which are derived from the C terminus of AA and DHA, were more significantly formed than SUD and PRD; however, no significant difference of adduct level was found between the 7- and 27-week-old rats.

Identification of HED as a Potent Inducer of Neuronal Apoptosis—In recent years, several dopamine oxidants and dopamine-modified adducts have been reported, such as neuromelanin (22), aminochrome (23), 6-OHDA (24), and 5-S-systhynildopamine (19), in which 6-OHDA has been generally known as a potent neurotoxin (25–27). We hypothesized that some of these DHA- and AA-derived dopamine adducts could cause neuronal cell death. To test this hypothesis, the effect of these dopamine adducts on the cell viability in SH-SY5Y cells was studied. After treatment with 100 μM of the sample for 24 h, among the tested dopamine adducts, HED and PRD induced about 80 and 30% of the cell death, respectively. On the other hand, SUD and GLD had almost no influence on the cell viability (Fig. 4*A*), suggesting that the death of SH-SY5Y cells was induced only by the C terminus-derived adducts and not by the C terminus-derived adducts. Of interest, two HED analogs, nonanoyl dopamine (NOD) and lauroyl dopamine (LAD), which were synthesized in this study and characterized by more carbons than HED in the methyl terminus, also showed

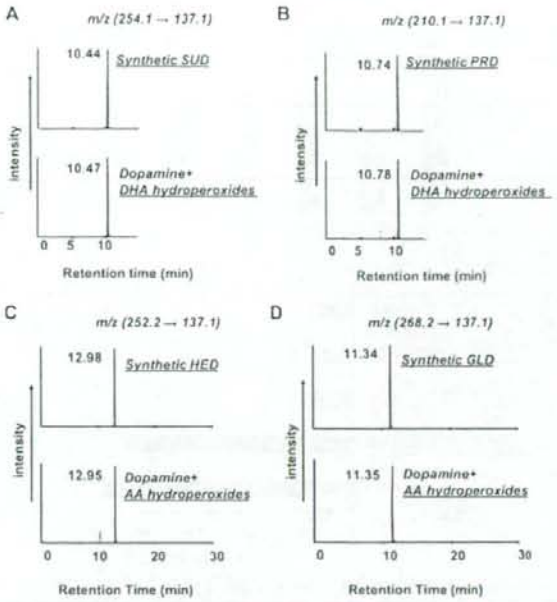

FIGURE 2. **HPLC-MS/MS analysis of the dopamine adducts formed during the reaction of dopamine with oxidized DHA and AA hydroperoxides.** Dopamine (2 mM) was incubated with lipid hydroperoxides (10 mM) in 0.1 M phosphate buffer (pH 7.4) at 37 °C. Shown is selected ion monitoring of the transitions from *m/z* 254 (*A*), 210 (*B*), 252 (*C*), and 268 (*D*) to *m/z* 137 for SUD, PRD, HED, and GLD, respectively. *Top panels*, authentic dopamine adduct; *bottom panels*, reaction mixture of DHA- or AA hydroperoxides with dopamine.

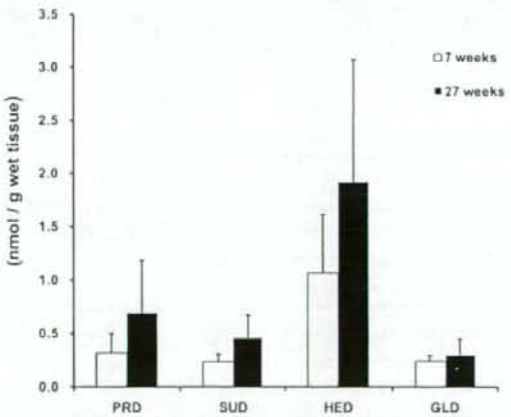

FIGURE 3. **Formation of the dopamine adducts *in vivo*.** The levels of dopamine adduct formed in rat brain were determined by HPLC-MS/MS (data are shown as the means ± S.D. (n = 5)).

a significant toxicity to SH-SY5Y cells (Fig. 4*B*), suggesting that the number of carbons in the C terminus-derived dopamine adducts might be associated with the adduct-induced cell death.

Remarkably, HED was a potent inducer of SH-SY5Y cell death compared with SUD, PRD, and GLD. Because the main cause of neuronal cell death has been postulated to be apoptosis, we then characterized whether HED-induced cell death in SH-SY5Y cells includes apoptosis. As shown in Fig. 5*A*, the

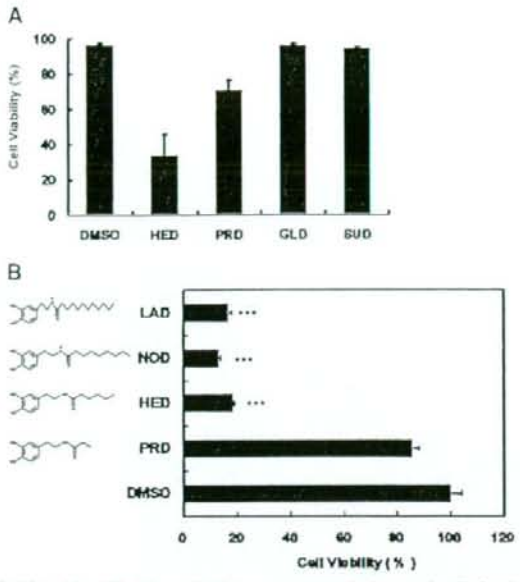

FIGURE 4. **Identification of HED as a potent inducer of cell death in SH-SY5Y cells.** The cells were exposed to 100 μM sample for 24 h. Cell viability was measured by the MTT assay. In the MTT assay, the data are expressed as percentages of control culture conditions. *A*, potential comparison of cell death induction by DHA- and AA-derived dopamine adducts. *B*, effect of carbon numbers in C terminus in the structure of dopamine-derived adducts to cell viability in the cells (data are shown as the means ± S.D. (n = 3); ***, indicates $p < 0.001$).

exposure to HED led to a dose-dependent decrease in the viable cells. When the SH-SY5Y cells were exposed to 10 μM HED for 4 h, the fragmented nuclei were found in cells exhibiting the typical morphological features of apoptosis (Fig. 5*B*). In addition, the gel electrophoresis of DNA from the SH-SY5Y cells exposed to HED also displayed nucleosomal DNA fragmentation (Fig. 5*C*). HED treatment also led to the time- and dose-dependent cleavage of PARP, resulting in the accumulation of the 85-kDa fragment and decreasing in the 116-kDa protein, as well as in the accumulation of the active caspase-3 (Fig. 5*D*), both of which are hallmarks of apoptosis. Moreover, the pretreatment with the caspase-3 inhibitor significantly prevented SH-SY5Y cells from HED-induced DNA fragmentation (Fig. 5*E*), providing further evidence that HED induced a caspase-3-mediated apoptotic cell death.

Regulation of HED-induced Apoptosis in SH-SY5Y Cells—We next investigated the signaling mechanism underlying the HED-induced apoptosis. It is well accepted that ROS generation is a key contributor to neuronal apoptosis induced by neurotoxin compounds (28). Hence, experiments were first carried out to assess ROS generation induced by the HED treatment and the possibility that the HED-induced apoptosis is mediated via ROS generation in SH-SY5Y cells. As shown in Fig. 6*A*, HED led to about a 3.5-fold increased ROS generation in the cells compared with the Me_2SO-treated cells, whereas the other three dopamine adducts, SUD, PRD, and GLD, had much less of an effect on the cells. Furthermore, after the HED treatment for 30 min, a dose-dependent increase in ROS generation was found by DCF fluorescence staining (Fig. 6*B*). The pretreatment with *N*-acetyl-L-cysteine, a potent antioxidant, clearly inhibited the PARP cleavage (Fig. 6*C*), indicating that ROS generation might be critically involved in the HED-induced apoptosis.

It is widely accepted that mitochondrial dysfunction may play very important roles in neuronal cell death (29). Here, we examined cytochrome *c* release from mitochondria in the cells, which are an important feature of mitochondrial change and a potent inducer of caspase-3 activation. As shown in Fig. 6*D*, dose- and time-dependent decreases of cytochrome *c* expression in the mitochondrial fraction were obviously observed in HED-treated cells.

Effect of Monoamine Transporter Inhibition on HED-induced Apoptosis and ROS Generation—Monoamine transporters including the dopamine transporter (DAT), norepinephrine transporter (NET), and 5-HT transporter (5-HTT), which are of fundamental importance for proper signaling between neurons, have been reported to associate with experimental neurotoxins-induced toxicity (30). HED possesses a dopamine-based chemical structure; therefore, in this study we hypothesized that the above-described HED cytotoxicity that occurred in the SH-SY5Y cells might be mediated by some monoamine transporters. To evaluate this hypothesis, we used the monoamine transporter inhibitor to investigate the effect of DAT, NET, and 5-HTT on HED-induced apoptosis and ROS generation. As shown in Fig. 7*A*, the pretreatment with both GBR12909 and imipramine, the inhibitors of DAT and NET/5-HHT, respectively, clearly inhibited the occurrence of the HED-induced PARP cleavage and active caspase-3 expression in the SH-SY5Y cells. Furthermore, ROS generation by HED was also found to be suppressed in these two inhibitor-pretreated cells. The result that both monoamine transporter inhibitors showed markedly inhibitive effect on the HED-induced apoptosis and ROS generation suggested that HED might be primarily transported into the SH-SY5Y cells by the monoamine transporters and inflicted damage on the cells.

Influence of HED to NIH-3T3 Cell Lines—To characterize whether the HED-induced cytotoxicity is specific to neuronal cells, we investigated the effect of HED on apoptotic cell death and ROS generation in mouse embryonic fibroblast NIH-3T3 cells in comparison with that of the SH-SY5Y cells. A dose-dependent analysis revealed that HED led to no apoptotic cell death in the NIH-3T3 cells based on Hoechst 33258 and PI nuclear staining (Fig. 8*A*). A further quantitative analysis of the apoptotic cells by flow cytometry also indicated a significant apoptosis in SH-SY5Y cells, whereas not in the NIH-3T3 cells (Fig. 8*B*). Moreover, no ROS generation was found in the HED-treated NIH-3T3 cells; on the other hand, the HED analogs, NOD and LAD, also induced only a slight ROS generation in the NIH-3T3 cells (Fig. 8*C*). These data and the fact that monoamine transporter is absent in NIH-3T3 cells suggest that the HED-induced cytotoxicity might be specific to neuronal cells.

DISCUSSION

The nervous system is particularly vulnerable to the deleterious effect of ROS, and one of the main reasons is that the brain contains high concentrations of polyunsaturated fatty acid that are highly susceptible to lipid peroxidation (31, 32). In recent years, an increasing body of evidence suggests that oxidative

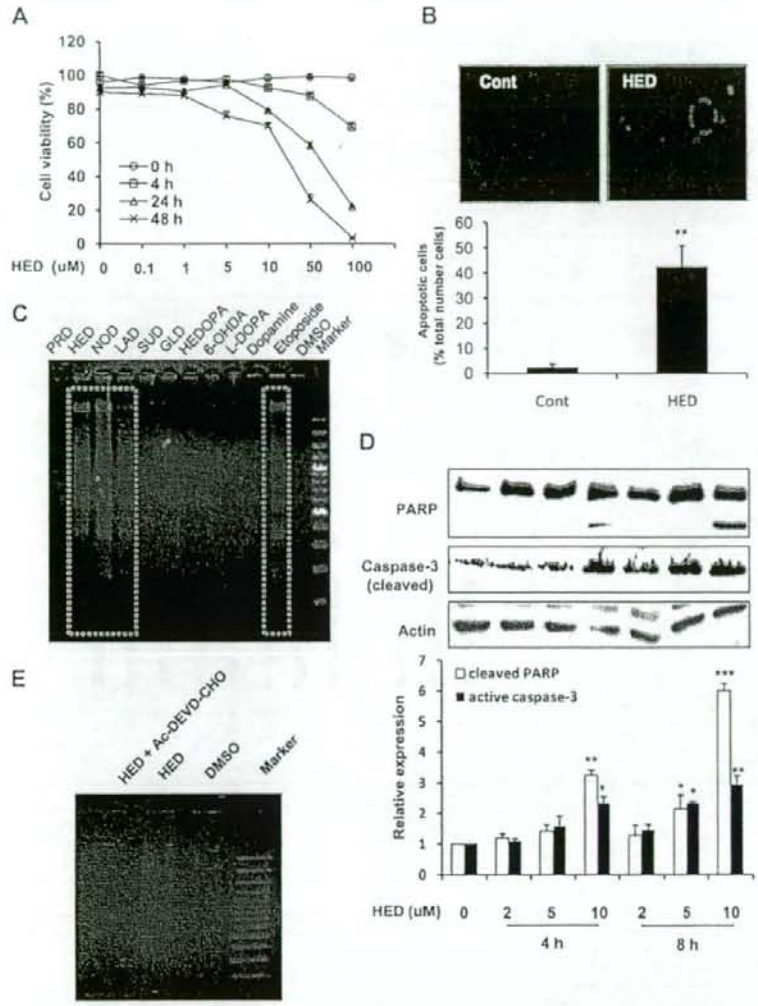

FIGURE 5. **Apoptosis induced by HED.** *A*, dose- and time-dependent cytotoxicity of HED. SH-SY5Y cells were exposed to 0–100 μM HED for different retention times. Cell viability was measured by the MTT assay. *B*, chromatin condensation in SH-SY5Y cells exposed to 10 μM HED. The cells were fixed with paraformaldehyde, stained with Hoechst 33258, and examined by fluorescence microscopy. *Upper left panel*, control (*Cont*) cells staining. *Upper right panel*, HED-treated cells staining. *Lower graph*, statistical analysis of apoptotic cells. *C*, DNA fragmentation in SH-SY5Y cells exposed to 25 μM HED or other samples for 12 h. Nucleosomal DNA fragmentation was visualized by agarose gel electrophoresis. *D*, PARP cleavage and active caspase-3 expression in SH-SY5Y cells exposed to 0–10 μM HED for 4 h and 8 h. The cleavage of PARP and expression of active caspase-3 were tested by Western blotting and statistically analyzed. *E*, effect of caspase-3 inhibitors on HED-induced DNA fragmentation. The inhibitor used was AC-DEVD-CHO. The SH-SY5Y cells were treated with 25 μM HED for 12 h in the presence or absence of inhibitor for 30 min. DNA fragmentation was visualized by agarose gel electrophoresis. All of the data are shown as the means ± S.D. ($n = 3$) (significantly different from control: * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$).

stress is pathologically involved in neurodegenerative disorders (33) including PD and Alzheimer disease. It is also generally accepted that lipid peroxidation, a central feature of oxidative stress, is an important reaction leading to oxidative damage in biomolecules, such as DNA and proteins (34–36). In the present study, we determined the formation of brain polyunsaturated fatty acid-derived dopamine adducts *in vitro* and *in vivo*. We also found that HED, an AA-derived dopamine adduct, significantly induced a monoamine transporter-mediated ROS generation and apoptosis in the SH-SY5Y cells. These data suggest that the DHA- and AA-derived dopamine adducts may be useful biomarkers, and their formation may be critically involved in the pathogenesis of Parkinson disease.

PD is one of the most common neurodegenerative disorders among the aged, and its pathological hallmark is the selective degeneration of dopaminergic neurons in the substantia nigra accompanied with the subsequent deficiency of dopamine in the brain areas. The etiology of PD remains unclear, but recently, mutation of the genes encoding α-synuclein and parkin was linked with familial PD. Additionally, dysfunction of mitochondria complex 1 and increase in oxidation of biomolecules were detected in dopamine neurons of postmortem brains of patients with idiopathic PD (37, 38). Lipid peroxidation, the central feature of oxidative stress, has been shown to increase in the PD brain, which is shown by such occurrences as increased malondialdehyde levels (39), HNE-modified proteins (40) and cholesterol lipid hydroperoxide (41), and lipoprotein oxidation in cerebrospinal fluid and plasma (42).

The sources of lipid peroxidation in the brain are thought to mainly originate from the peroxidation of DHA and AA because of their high contents in the brain relative to other organs (43) and highly unsaturated properties. In our previous reports, we have described that DHA and AA hydroperoxide, the primary products of fatty acid peroxidation, can universally react with primary amino groups to form amide linkage adducts including N^{ε}-(succinyl) lysine, N^{ε}-(propanoyl) lysine, N^{ε}-(hexanoyl) lysine, and N^{ε}-(glutaryl) lysine; however, through this study it is now recognized that DHA and AA hydroperoxides can also modify dopamine by an *N*-acyl-type adduct-formed reaction (Fig. 1).

Dopamine is a natural neurotransmitter in the brain, and its deficiency is a sign of Parkinson disease (44). Although the reason for dopamine loss is not fully understood, some considerations include dopaminergic neuron abnormalities, dopamine degradation by monoamine oxidase A, and auto-oxidation and

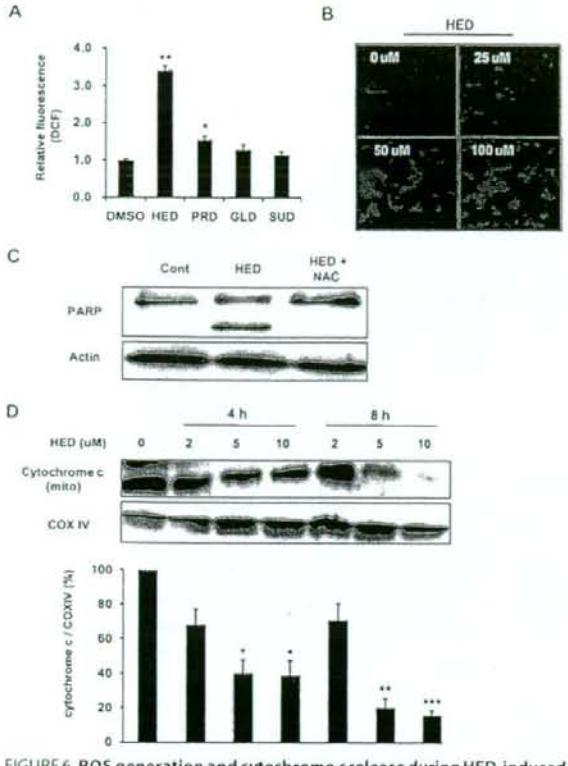

FIGURE 6. **ROS generation and cytochrome *c* release during HED-induced apoptosis.** *A*, comparison of ROS generation inducted by DHA- and AA-derived dopamine adducts. The SH-SY5Y cells were treated with 10 μM dopamine adducts for 30 min and exposed to H_2DCF-DA for 30 min. The fluorescence of DCF was measured by flow cytometer. *B*, dose-dependent ROS generation induced by HED. DCF fluorescence imaging was determined by fluorescence microscope. *C*, effect of antioxidant *N*-acetyl-L-cysteine on HED-induced PARP cleavage and accumulation of active caspase-3. 50 mM *N*-acetyl-L-cysteine was administrated in SH-SY5Y cells for 30 min before HED treatment. *D*, cytochrome *c* release induced by HED. The SH-SY5Y cells were treated with different concentrations of HED for 0, 4, and 8 h. The expressions of cytochrome *c* and cytochrome *c* oxidase IV (*COX IV*) in the mitochondrial fraction of HED-treated cells were assessed by Western blot. All of the data are shown as the means ± S.D. ($n = 3$) (significantly different from control: * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$. *DMSO*, dimethyl sulfoxide; *Cont*, control.

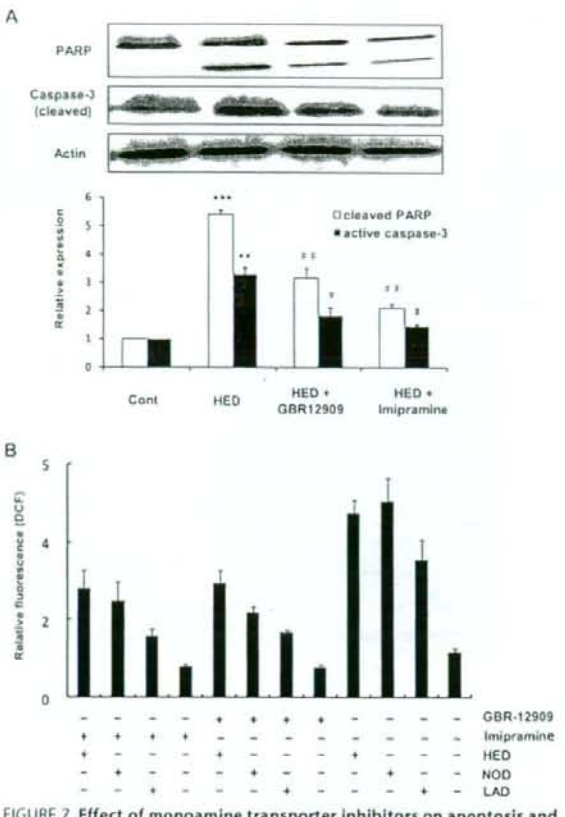

FIGURE 7. **Effect of monoamine transporter inhibitors on apoptosis and ROS generation.** The inhibitors used were GBR 12909 and imipramine for DAT and NET/5-HTT, respectively. 1 μM inhibitors were administrated in SH-SY5Y cells for 30 min before drug treatments. *A*, effect of monoamine transporter inhibitors on HED-induced PARP cleavage and accumulation of active caspase-3. Cleaved PARP and the expression of active caspase-3 were statistical analyzed. *B*, effect of monoamine transporter inhibitors on HED-induced and HED analog-induced ROS generation. All of the data are shown as the means ± S.D. ($n = 3$) (significantly different from control: *** indicates $p < 0.001$; significantly different from HED alone: # indicates $p < 0.05$, and ## indicates $p < 0.01$). *Cont*, control.

modification (17–19). The *in vitro* and *in vivo* detections of DHA- and AA-derived dopamine adducts established in this study (Figs. 2 and 3) may indicate an additional clue to the causes of dopamine deficiency in PD. Although the level of the dopamine adducts was not obviously increased in the 27-week-old rat brain compared with the 7-week-old rat brain, 27 weeks represents only middle age for a rat, and the level of basal oxidative stress is increased with age (45–47); therefore, further study should confirm these adduct formations in the brain using aging model rats such as rats 1 year old and more and also PD model animals.

Dopamine-derived metabolites have been reported to inflict damage on neuronal cells (48). For example, 6-OHDA, a hydroxylated analog of dopamine, has been demonstrated to induce apoptosis in several neuronal cell lines (49–52). In addition, dopamine auto-oxidation generating dopamine quinone can react with protein sulfhydryl groups leading to structural modifications of proteins and reduced levels of glutathione (53). In the present study, we found that HED, an AA-derived dopamine adduct, caused significant cell death in SH-SY5Y cells (Fig. 4*A*). Furthermore, the events including DNA fragmentation, chromatin condensation, PARP cleavage, and accumulation of active caspase-3 (Fig. 5) suggest that HED-induced cell death includes apoptosis. The precise mechanisms regulating apoptotic events in neuronal cells remain largely unclear; however, high levels of ROS generation and the increases in the mitochondrial permeability appear to be common occurrences in many forms of apoptotic neuronal cell death. The finding that HED induced a significant ROS generation and that *N*-acetyl-L-cysteine pretreatment clearly blocked the apoptosis suggests that ROS generation is an essential trigger for HED-induced apoptosis in the SH-SY5Y cells. The source of ROS generation has not been identified; however, the catechol ring is kept in the structure of HED like dopamine and 6-OHDA; therefore, the catechol oxidation might be one of the

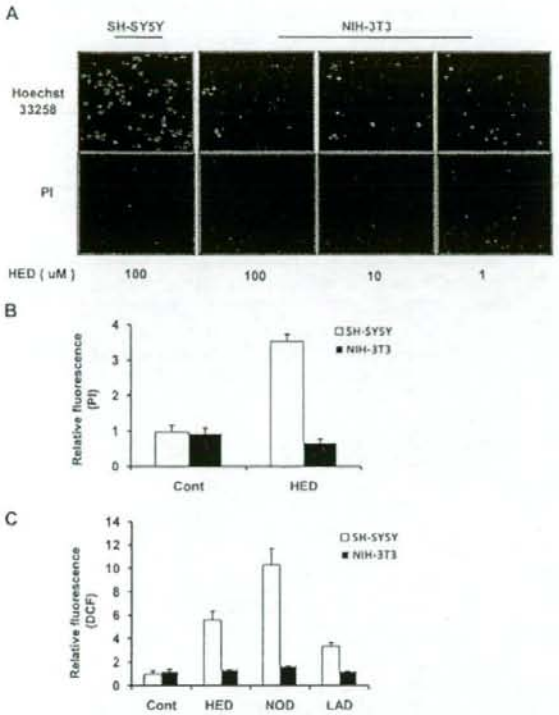

FIGURE 8. **No cytotoxicity was induced by HED in NIH3T3 cells compared with in SH-SY5Y cells.** *A*, apoptotic cells imaging. NIH3T3 cells were treated with different concentrations of HED for 12 h. PI and Hoechst staining were performed by fluorescence microscope. *B*, numbers of apoptotic cells. The apoptotic cells were analyzed by PI staining by using a flow cytometer. *C*, ROS generation. The fluorescence of DCF was measured by flow cytometer. The data are shown in *B* and *C* as the means ± S.D. ($n = 3$). *Cont*, control.

important causes for ROS generation in the HED-treated SH-SY5Y cells. The regulation of neuronal apoptosis is generally characterized by several signaling mediators such as p53, Bcl-2 family proteins, and cytochrome *c* release (54). A significant release of cytochrome *c* from mitochondrial fraction in HED-treated SH-SY5Y cells was found (Fig. 6), suggesting that the apoptosis may be critically mediated via a mitochondrial abnormality; however, the changes of Bcl-2, Bax, and phosphorylated p53 expression were not seen (data not shown); therefore, the upstream regulators of mitochondrial abnormality remain to be elucidated in further studies.

Monoamine transporters are of fundamental importance for proper signaling between neurons. Plasma membrane transporters, the major subclass of intracellular transporters (55), include the DAT, NET, and 5-HTT. In this study, pretreatment with inhibitors of DAT, NET, and 5-HTT significantly suppressed ROS generation and apoptosis events induced by HED (Fig. 7). In the case of 6-OHDA, similar to HED, a high affinity for several catecholaminergic plasma membrane transporters, such as DAT and NET, is also essential for its entrance into the neuronal cells to inflict damage. The dependence of monamine transporter is considered to be due to a structural similarity between the monamine transporter and dopamine and norepinephrine. The necessity of the monoamine transporter in HED-induced cytotoxicity was further demonstrated by the result that HED could not induce apoptotic cell death and ROS generation in the monoamine transporter-absent NIH-3T3 cells (Fig. 8), which also indicates that HED may selectively induce cytotoxicity in different cell lines.

PRD, HED, NOD, and LAD, which have 3, 6, 9, and 12 of carbons in the methyl terminus based on the dopamine structure, respectively, caused apparent cell death and ROS generation in SH-SY5Y cells in the order of PRD < HED ≈ NOD ≈ LAD (Figs. 4*B* and 7*B*), which suggests that the specific carbon number in the C terminus might be required for the dopamine adduct-induced cytotoxicity. On the other hand, SUD and GLD, which are C terminus adducts, showed no toxicity to the SH-SY5Y cells. Moreover, to further confirm the difference of the cytotoxicity between the C terminus and the C terminus in dopamine adducts, we synthesized a compound named hexanoyl dihydroxyphenylalaine (HEDOPA), which structurally distinguishes HED as HEDOPA that possesses a more C terminus than HED, by the reaction of hexanoly acid with dihydroxyphenylalanine (L-DOPA), which is the precursor of dopamine. Following HEDOPA treatment in the SH-SY5Y cells compared with HED, HEDOPA did not alter the viability and induce ROS generation in the cells (data not shown). These results reveal that the C terminus may structurally inhibit the transport of dopamine adducts into the cells and subsequently block the induction of cytotoxicity.

The formation of the dopamine adducts in the study are established by free polyunsaturated acid. In fact, either DHA or AA is located almost exclusively in the SN2 position of phosphoglycerides found in the neural cell membranes (56, 57); however, free fatty acid levels are reported to increase with aging because of an increasing degradation by phospholipase A_2 (58–60), which selectively acts on phosphoglycerides (61). DHA is the most enriched polyunsaturated fatty acid in the brain, and it has been implicated that DHA concentration is decreased in Alzheimer disease brain (62); hence, the DHA-derived dopamine adducts formed in this study may be useful biomarkers for not only PD but also Alzheimer disease.

In summary, we synthesized four dopamine adducts derived from DHA and AA and revealed the *in vivo* formation during the reaction of lipid hydroperoxides with dopamine. We observed HED, an AA-derived dopamine adduct, as a potent neurotoxin based on the significant induction of ROS generation and apoptosis in human neuroblastoma SH-SY5Y cells. The mechanism of HED-induced apoptosis has not been fully established in this study; however, it seems to be mediated by ROS generation, mitochondrial abnormalities, and monoamine transporter. The HED-induced cytotoxicity is confirmed by an *in vitro* experimental system in this study, and further studies showing the existence and the cytotoxicity in human subjects are needed.

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Original Article

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Oxidation of Polyunsaturated Fatty Acids Induces Protein Oligomerization and May Initiate Neuronal Death Process in Parkinson's Disease

Masayo Shamoto-Nagai[1], Wakako Maruyama[1,*], Yoji Kato[2], and Toshihiko Osawa[3]

[1]Department of Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan
[2]Graduate School of Human Science and Enviroment, University of Hyogo, Himeji, Hyogo, Japan
[3]Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Nagoya, Aichi, Japan

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[1]Department of Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan
[2]Graduate School of Human Science and Enviroment, University of Hyogo, Himeji, Hyogo, Japan
[3]Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Nagoya, Aichi, Japan

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Summary **Docosahexaenoic acid (22:6n-3, DHA) is one of the most popular long chain polyunsaturated fatty acids derived from fish oil. DHA is essential fatty acid for human and dependent on oral intake. It is well known that DHA is rich in the brain and the retina where it exists as the components of cellular membrane. Administration of DHA to the rodents improves their brain functions and it is suggested DHA in the membrane plays a role in the neuronal system. In addition, DHA is a potent antioxidant, but simultaneously, is easily oxidized and produces toxic lipid peroxide. Conformational change and abnormal aggregation of protein are commonly observed features in the neurodegenerative disorders, such as Parkinson disease (PD). In PD, aggregation of α-synuclein (α-Syn), called Lewy body is observed, but the mechanism of protein aggregation has not been elucidated. The effect of lipid peroxide derived from DHA on oligomerization of α-Syn was investigated. Oxidation of DHA enhanced oligomerization of α-Syn and adduct formation of lipid peroxde to α-Syn was identified. These results suggest that oxidative stress induced by ageing may enhance oxidation of DHA in the cellular membrane, then, initiate toxic oligomerization of proteins in PD.**

Key Words: **lipid peroxidation, parkinson's disease, polyunsaturated fatty acid, protein aggregation**

Introduction

Long chain polyunsaturated fatty acid (PUFA), are enriched in the nervous system. Docosahexaenoic acid (22:6n-3, DHA) is one of the most abundant PUFA in the brain and retina, and is component of phosphatidylethanolamine and phosphatidylserine in the celluler membrane. Administration of DHA to the rodents improves their brain functions [*1*] and it is suggested DHA in the membrane plays a role in the synaptic plasticity and signal transduction systems in the neuronal cells [*2–4*]. In the human, lipids orally administrated are reconstructed through β-oxidation and elongation reaction mainly in the liver after digestion and absorption. However, human can not synthesize enough amount of DHA, so, the intake of DHA from food, mainly fish oil, is essentially required (essential fatty acid). Brain DHA is derived from the circulating plasma pool and delivered to the brain across the Blood-Brain-Barrier. In the human brain, the amount of n-6 PUFA, such as arachidonic acid (AA) is estimated to be 17.1% of the total fatty acids, and on the other hand, the amount of n-3 PUFA, such as DHA is estimated to be 9.7%. The concentration of n-3 PUFA is higher than that of plasma, so that, the existence of specific

*To whom correspondence should be addressed.
Tel: +81-562-46-2311 (ex 5551) Fax: +81-562-46-8264
E-mail: maruyama@nils.go.jp

transporter for DHA is suggested in the neural cells and astrocytes [5]. DHA is known to be a potent antioxidant, but simultaneously, it is easily oxidized and produced toxic lipid peroxide. These toxic lipid peroxide and their products, such as aldehyde may form adduct with the proteins to induce degenerated proteins with abnormal conformation. The proteins, which associated with cell membrane, such as cell-surface receptor, membrane anchoring protein, or functionally transmembrane protein are the candidates. The oxidized products are degradated by ubiquitin-proteasome system or autophagy, but according to ageing, the accumulation of these abnormal proteins might perturb homeostasis to induce neuronal death.

Parkinson's disease (PD) is the second common neurodegenerative disorder and affects 1–2% of aged population over 60 years old. The pathogenesis of PD has not been clarified, but aggregation of protein with abnormal conformational change is commonly observed features in the neurodegenerative disorders. In PD, degeneration of dopamine neuron in the substantia nigra and the existence of Lewy bodies (LB), are diagnostic pathological features. α-Synuclein (α-Syn) is the main component of LB and is known to exist as membrane-bound form by association with PUFA. The mutation of A53T, A30P, and E46K, or triplication of α-Syn gene have been identified in early-onset familial PD [6–8]. It is suggested that accumulation of α-Syn is the cause, not the result of PD. In sporadic PD without α-Syn mutation, some post-translational modification of α-Syn may induce pathological process similar to familial PD. α-Syn is a 140-amino acid protein expressed ubiquitously in the neuron and accounts for 0.1–1% of brain cytosolic proteins. α-Syn is suggested to play many roles in nervous system, including regulation of synaptic vesicle mobilization, chaperone activity, modulation of dopamine transporter and dopamine biosynthesis [9–11]. In this paper, the oxidative modification of α-Syn by lipid peroxide and aldehyde derived from PUFA was examined. The possibility that lipid peroxidation in the membrane-composing PUFA and adduct formation with α-Syn was investigated. The results are discussed in relation to the role of oxidative stress in brain ageing in the pathogenesis of PD.

Materials and Methods

PUFA is oxidized and produce lipid peroxides, then, form adducts with lysine residues in the proteins. DHA is oxidized and split to produce succinyl-lysine adduct (SUL) with carbonyl terminal and propanoyl-lysine adduct (PRL) with amino terminal, respectively. AA is also oxidized, to produce glutaroyl-lysine (GLL) with carbonyl terminal and hexanoyl-lysine (HEL) adduct with amino terminal. The antibodies of these 4 oxidized PUFA products, SUL, PRL, GLL and HEL are prepared as reported previously [12]. Recombinant α-Syn is purachased from BIOMOL International, L.P. (Plymouth Meeting, PA.). DHA, AA and oleic acid donated from CAYMAN CHEMICAL (Ann Arbor, MI) and stearic acid are Nu-chek prep, Inc. (Elysian, MN). Other chemicals are from WAKO finechemical (Osaka, Japan).

Recombinant α-Syn (2 μM) was co-incubated with long chain fatty acids namaly DHA, AA, oleic acid or stearic acid (1–10 mM) for 3 to 7 days. The reaction products were separated by SDS-PAGE and were analyzed by Western blotting using anti-α-Syn antibody. The sample incubated with DHA or AA, the production of oxidized PUFA was examined also, using antibodies described above. The effects of antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), on oligomerization of α-Syn was examined also.

We examined whether DHA is neuroprotective or neurotoxic using human clonal neuralblastoma SH-SY5Y cells. Free DHA (0–100 μM) or DHA (0–100 μM) pre-incubated with 4 folds concentration of Bovine serum albumin (BSA), which acts as lipid carrier protein was added to the culture medium in the presence or absence of tocopherol (100 μM) for 2 days in 5% CO_2–95% atmosphere at 37°C. Culture medium was COS-Medium 001 (COSMOBIO, Japan) without serum. Cell death was estimated using LDH assay according to manufacture's procedure. The cells were mechanically harvested and gathered, then homogenized in RIPA Buffer, then centrifuged at 10,000 × g for 15 min. The supernatant and pellet were used as soluble and insoluble fraction, respectively. The amount of lipid peroxides in the soluble and insoluble fraction of the cells was estimated by spectrofluorophotometer RF-5300 PC (SHIMADZU, Japan) with excitation at 365 nm and emission at 440 nm.

Results

α-Syn oligomerization was found to be enhanced by the existence of DHA and AA in a dose- and time-dependent way, but not that of oleic acid and stearic acid. These results indicate that the existence of double-bounds in long chain fatty acid is essential for enhancement of α-Syn oligomerization. α-Syn oligomers were found to be positive for SUL and PRL in the sample incubated with DHA, and GLL and HEL in that with AA. BHA and BHT were found to reduce α-Syn oligomerization enhanced by DHA and AA. It was indicated lipid peroxide produced by PUFA formed adduct with α-Syn, then oligomerization and aggregation of the protein.

Free DHA at the concentration higher than 20 μM was found to be cytotoxic to SH-SY5Y cells. Antioxidant tocopherol could not prevent the toxicity of free DHA. DHA pre-incubated with BSA (DHA-BSA) was less toxic than free DHA and tocopherol inhibited the toxicity by DHA-BSA completely.

The amount of PRL, which reflects the level of the proteins conjugated with lipid peroxide derived from DHA, was found to increase in the soluble and insoluble fractions in the cells treated with DHA-BSA. On the other hand, tocopherol reduced the level of PRL significantly.

These results indicated that the cytotoxic effect of DHA-BSA was induced by the conjugation of oxidation product of DHA with cellular proteins. The cytotoxicity of free DHA should be ascribed to its detergent activity.

Discussion

In this paper we demonstrated that DHA enhanced oligomerization of α-Syn through adduct formation with lipid peroxide derived from DHA (SUL and PRL) with α-SYN *in vitro*. Using cell culture system, DHA-BSA, showed weak cytotoxic effect on neural cells and adduct formation of the cellular proteins with PRL was also identified. Antioxidant inhibited cytotoxic effect simultaneously. These results suggest that DHA, which has been believed to be neuroprotective, may become neurotoxic in the condition with increased oxidative stress, such as neurodegenerative disorders.

The increased lipid peroxidation of the membrane is suggested to play an important role in the vicious process in ageing. Lipid peroxides in the membrane initiates sequential reaction of PUFA oxidation to increase the amount of oxidized fatty acids, to decrease the membrane fluidity and perturb the function of the proteins associated with cellular membrane. In PD, where dopamine neuron degenerated and membrane-associated protein, α-Syn is aggregated, increased oxidative stress, mitochondrial dysfunction and impaired ubiquitin-proteasome system were observed. Oxidation of PUFA, especially DHA may decrease the binding capacity of α-Syn to the membrane. α-Syn released from the membrane to the cytosol can't stabilize its α-helix structure no more. In addition, lipid peroxide may directly make adduct with α-Syn and induce protein oligomerization as shown in this paper.

Epidemiological study suggests that the intake of fish oil and vegetable decrease the risk of neurodegenerative disorders, such as Alzheimer's disease and PD [*13*]. However, intervention of neurodegenerative disorders by DHA has not been successful. The results of this paper that in the brain of neurodegenerative disorders, where oxidative stress increased, DHA may neurotoxic. DHA may be effective when it is administrated not after but before the onset of the disease. In addition, intake of the food-derived antioxidant such as polyphenol, which can prevent the oxidation of DHA may increase its usefulness (Fig. 1). The further investigation to clarify the effect of food-derived DHA and polyphenol using clinically available biomarker is now under the way.

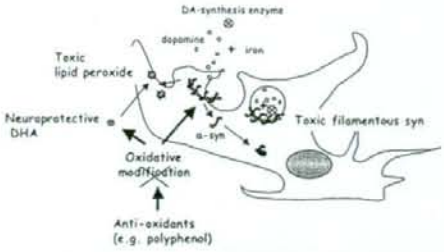

Fig. 1. DHA is generally neuroprotective, but potentially neurotoxic when the oxidative stress is increased. Under the oxidative stress, DHA may produce toxic lipid peroxide and produce toxic protein adducts. Polyphenol or other food-derived antioxidants might prevent the toxicity of DHA by reducing the oxidative stress.

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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，ペルオキシラディ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を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．

＊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．

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野菜(植物性食品)摂取の効果

国立長寿医療センター研究所 老年病研究部
丸山和佳子 *Maruyama, Wakako*

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（生体の維持に必須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B_{12}，B_6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（Aβ）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，Aβ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が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	ブロコッリー，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Aβの蓄積が認められる．近年，Aβに対する免疫を賦活することで脳内のAβ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Aβ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，Aβ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Aβの合成を抑制したり，あるいはAβの凝集を抑制することで脳内Aβ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

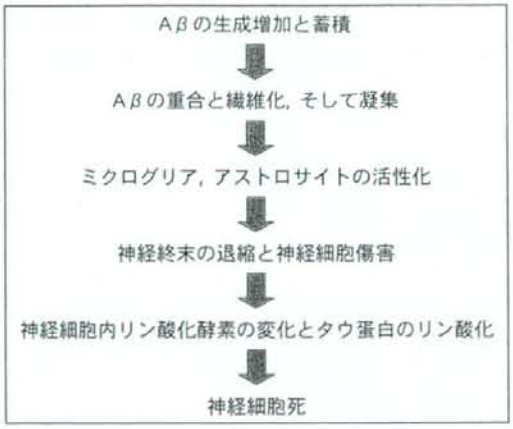

図1　アルツハイマー病のアミロイド仮説
最初になんらかの原因（遺伝子変異，老化，酸化ストレスなど）でベータアミロイド(Aβ)の増加と蓄積が起こり，細胞死のカスケードが起動される．

表2　疫学研究と介入研究の差

	介入研究（RCT）	疫学研究
評価対象	精製標品（薬剤）	複合品（食品），ライフスタイル
期間	短期（数カ月―数年）	長期（数十年）
対象	疾病患者	コホート （一般地域住民のことが多い）
評価基準	疾病の進行抑制，治癒	疾病発症頻度の抑制

い．認知症を発症した患者を同質な2群に分け，有効と思われる成分を投与された群と偽薬（プラセボ）を与えられた群で症状の進行を比較する（randomized controlled study = RCT）（表2）．

これまでの研究では，疫学研究では野菜摂取の抗認知症効果を示す多くの結果が報告されている[6,7]一方，介入研究のほとんどは失敗に終わっている[8]．この原因は，食品由来成分は薬剤のような強力な作用をもたないため，短期的な治療効果というより長期的な予防効果を検討するための研究デザインが必要なためではないかと考えられる．

野菜由来の微量成分の抗認知症作用のメカニズム

■抗酸化作用

アルツハイマー病などの老年性認知症の最大のリスクファクターは老化である．“老化のフリーラジカル説”[9]は広く受け入れられている概念ではあるが，その実態はなんであろうか．酸化ストレスにより生体を構成する分子，たとえば核酸，蛋白質，脂質などが酸化修飾を受け，構造変化をきたしたり，機能不全を起こす．とくに，脳神経のような分裂能力の乏しい細胞ではこのような酸化修飾分子を新しい細胞をつくること（再生）により排除できないため異常な構造をもつ分子が蓄積しやすい．たとえばアルツハイマー病の原因にかかわるAβの蓄積には脳内の酸化修飾分子の蓄積や，酸化ストレスの亢進が関与している可能性がある．ビタミンC，E，ポリフェノール類は抗酸化作用をもち，抗老化，抗老年病効果をもつと期待されているが，介入研究，とくにサプリメントの摂取が有効であったとの証明はなされていない．

■遺伝子発現制御作用

近年，食品由来成分，とくにポリフェノール類であるレスベラトロール[10]，カテキン[11]などが遺伝子の発現を制御し，老化，老年病を抑制することが報告され，注目を集めている．レスベラトロールは赤ワインに含まれ，昔から“フレンチパラドックス（フランス人はアルコール摂取量が多いのに動脈硬化性疾患が少ない）”の原因ではないかとされてきた．レスベラトロールの働きとして抗酸化作用のほかに，寿命遺伝子であるsirtuin familyを活性化することが報告され，さらに実験動物で寿命を延長させたり，インスリン耐性を増強したりすることが見出された．しかし，ヒトに対する長寿作用は確認されておらず，さらに，日本人はアルコール耐性が低いのでワインの多量摂取によるアルコール毒性の危険が高いため勧められない．

一般患者への指導はどうあるべきか

現在，科学的に実証された“認知症を防ぐ食生活”は存在しない．しかしながら疫学的研究，動物実験ともに適正な量の野菜の摂取が認知症予防に有効である可能性を示唆している．また，とくに認知症患者さんのなかには偏った食生活

を続けており，他からの指導，介入が必要な方がおられることも事実である．このような患者さんに対し，規則正しい食生活と適切な量の野菜の摂取を促すことは，摂取食品そのものの改善に加え，生活習慣全般の改善により認知症の予防あるいは進行防止につながる可能性がある．

おわりに

医食同源とは日本古来からの哲学であり，超高齢化社会を迎えた現在，予防医療，統合医療の重要性が高まっている．認知症を予防する食生活を科学的に検証することが今後の課題である．

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砂糖(甘い菓子類)摂取の影響

自治医科大学附属さいたま医療センター　神経内科
大塚美恵子　*Otsuka, Mieko*

keyword
アルツハイマー病，高インスリン血症，
インスリン抵抗性，砂糖，菓子類

はじめに

2型糖尿病（T2DM）は世界的に急速に増加しており，全体的な運動不足と肥満の蔓延が拍車をかけている．T2DMはもはや中高年のたんなる疾患ではなく若年層においても現在，将来の健康を脅かす深刻な疾患とみなされている[1]．多くの疫学調査でT2DMはアルツハイマー病（AD）の発症と関連があるとされており[2,3,4]，さらに最近糖尿病をともなわない高インスリン血症も危険因子として注目されている[5]．

本稿では，AD患者の耐糖能異常と高インスリン血症の実態，食行動のなかでも砂糖（甘い菓子類）摂取との関連について調査した結果を中心に述べてゆく．

インスリンとアルツハイマー病に関する知見

インスリン受容体は脳に広く分布しており，とくに海馬に集中している．また，Frolichらの報告[6]ではAD患者の脳内インスリン受容体濃度が健常対照者に比較して増加している．これはインスリンシグナリングの欠乏を代償的に高めようとしているためと解釈されている．さらに，インスリン分解酵素Insulin-degrading enzyme（IDE）はアミロイドベータ蛋白(Aβ)の分解と除去に関連があり[7]，IDEはインスリンとの親和性が強いため高濃度のインスリンはIDEによるAβの分解を抑制し[7]，神経原線維を構成する重要な要素の一つであるタウ蛋白(τ)のリン酸化を増すとも報告されている．このようにインスリン調節異常はAβ沈着やτのリン酸化を増す[8]ことでAD発症にかかわっていると考えられる．したがって，インスリンとインスリン抵抗性の研究は，T2DMとADの関連を解明するのに期待される分野とみなされている．

アルツハイマー病患者の栄養学的問題点

ADと栄養の問題は糖尿病やインスリンに関する問題以外に，現在のところ以下の点に絞られてきている．まず，野菜・果物の摂取はADを予防し，ビタミンE，ビタミンCなどの抗酸化ビタミンが注目されている[9]．つぎに，魚の摂取はADを予防し，魚油に含まれるドコサヘキサエン酸(DHA；22：6 n-3)やエイコサペンタエン酸(EPA；20：5 n-3)などのn-3系多価不飽和脂肪酸（PUFA）の役割が注目されている[10]．第3に動脈硬化の危険因子である高ホモシステイン血症がADでも認められ，ビタミンB_6，ビタミンB_{12}，葉酸の欠乏との関連が注目されている．これらは酸化ストレス，慢性炎症，血管

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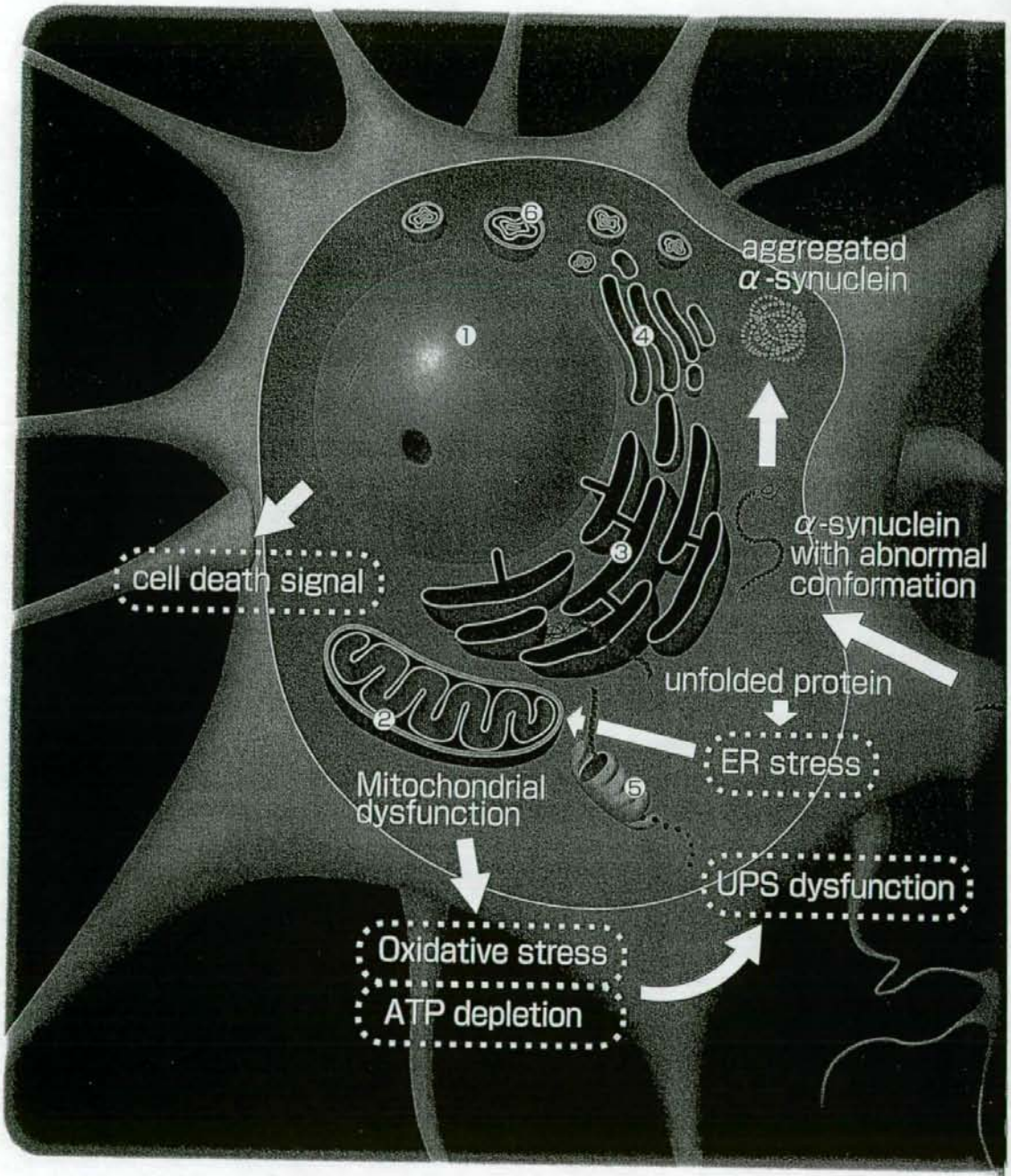
パーキンソン病の

Visual View

丸山和佳子
国立長寿医療センター 研究所老年病研究部 部長



パーキンソン病の病態と成因

近年多くの単遺伝子変異に由来するパーキンソン病（PD）が報告され，その病因が解明されつつある。しかし，PD のなかで 90%以上を占める孤発性 PD の疾患の原因はいまだに不明であり，老化に伴う神経細胞の機能異常が重要な役割を果たしている可能性がある。