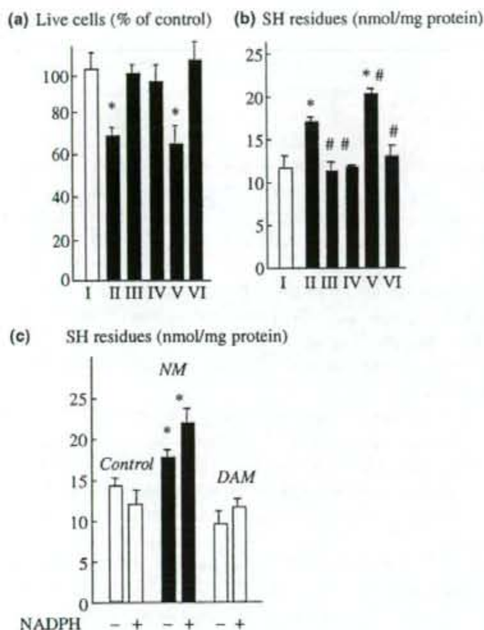

Fig. 6 Effects of antioxidants and NADPH on the NM-induced apoptosis and SH increase. (a) Effects of antioxidants on cell death induced by NM. The wild cells were treated without (I, control) or with NM alone (II) or NM in the presence of DFX (III, 1 μM), SOD (IV, 100 U/mL), catalase (V, 300 U/mL), and EGCG (VI, 10 μM) 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 μM), SOD (III, 100 U/mL), catalase (V, 300 U/mL), and EGCG (VI, 10 μM) 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 μg/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, $p < 0.05$.

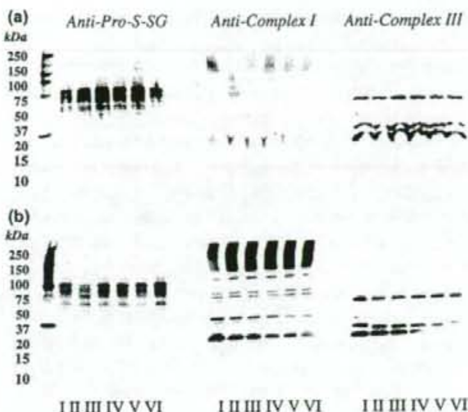

Fig. 7 Effects of NM on S-glutathionylated protein in mitochondria. Mitochondria were prepared from the wild cells and treated without (I, control), or with 10 μg/mL NM (II), P-K NM (III), DAM (IV), Cys-DAM (V), or 100 μM dopamine (VI) at 37°C for 2 h. The samples were washed with PBS, and subjected 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.

previous results that DAM increased ROS–RNS and induced cell death in neuronal SK-N-SH cells, whereas NM protected the cells from hydroxyl radicals produced by the Fenton reaction (Li *et al.* 2005). Melanosomes containing eumelanin and pheomelanin have oxidation potentials of −0.2 and +0.5 V, respectively (Samokhvalov *et al.* 2005). Pheomelanin in NM and Cys-DAM may reduce disulfide bonding and release GSH or cysteine from the mixed disulfide bonding and increases SH content in mitochondria, while eumelanin and DA oxidatively modify SH residues. These results are relevant with the fact that NM has the surface oxidation potential different from synthesized DAM (Bush *et al.* 2006).

The role of NM-associated protein was examined in concern to regulation of mitochondrial 'redox state,' which is related to many physiological and pathological phenomena of cells (Schafer and Buettner 2001; Maher 2006). Redox state depends on the reduction potential of redox pairs, GSH/GSSG, reduced/oxidized thioredoxin [$Trx(SH)_2$/TrxSS], and NADPH/$NADP^+$. GSH/GSSG pool is the largest in cells and a major indicator of the redox state. Most glutathione is localized in the cytosol, but there is a GSH pool in mitochondria, which accounts for about 15% of the total (Meister 1995). GSH reduces thiol modifications of disulfide bonding and thioesters, and it is a substrate of protein *S*-glutathionylation. *S*-Glutathionylation is reversible and transitional in cytosol, where mM order of GSH and ascorbic acid are present, but in mitochondria with high oxidative environments (Table 2), *S*-thiolated proteins are more stabilized and detected in complex I, as

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reported here and by Taylor *et al.* (2003). Glutathionylated $NADP^+$-dependent isocitrate dehydrogenase was detected in brains from a PD model of MPTP-treated mice (Kil and Park 2005). Under oxidative conditions, actin, glyceraldehyde-3-phosphate dehydrogenase, protein kinase, heat-shock protein 27, protein-tyrosine phosphatase 1B, and protein kinase Cα are the substrate of *S*-thiolation (Eaton *et al.* 2002). In physiological conditions or mild oxidative stress, thiols in protein (PrS-SG) or GSH are reversibly modified into active intermediates, such as thiolate, sulfenate, and sulfenic acid, by NO, superoxide, hydrogen peroxide, and peroxynitrite (Klatt and Lamas 2000). The activated protein SH groups reacts with GSH, or *vice verse* activated GSH reacts with reduced protein SH to generate GSH-protein mixed disulfide (PrS-SG). Prolonged or intense generation of ROS–RNS irreversibly oxidized thiols into protein cysteic acid by sulfonation, which was detected in the brains of patients with PD and Alzheimer' diseases (Choi *et al.* 2005). Glutathionylation is reversed by glutaredoxin (GRX), other TRX, and protein disulfide isomerase (EC 5.3.4.1), and yields free SH in protein (Pr-SH) and GSH from PrS-SG. This reaction is recycled by TRX reductase (EC 1.6.4.5) or GSH reductase (EC 1.6.4.2) using NADPH as a cofactor.

Neuromelanin significantly reduces PrS-SG detected in mitochondria, especially in complex I (Fig. 7). In addition, NM dissociates high structure of complex I into the subunits, and the dissociation is reversed by reducing agents, suggesting that *S*-glutathionylation stabilizes the high structure of complex I under physiological condition. It may be reasonable to consider that SH group of NM functions as that in GRX, reduces the disulfide bonding in mitochondrial protein, release free GSH, or cysteine and exposes free protein SH. Only NM, but not Cys-DAM, reduces mitochondrial PrS-SG, indicating that NM-associated protein, not pheomelanin, affects the mixed disulfide bonding in mitochondria. The SH amounts in NM used for these experiments were less than 1% of the total SH content present in mitochondria, suggesting the 'catalytic' function of NM. NADPH increased SH release by NM, but did not affect SH content in control, suggesting that NM recycling by NADPH enhances the deglutathionylation in a similar way as GRX.

The mechanism underlying induction of apoptosis by NM requires further studies to be fully elucidated. NM activated 'intrinsic' apoptotic pathway, but Bcl-2 overexpression did not prevent cell death, even though Bcl-2 protects the cells against apoptosis induced by DA (Fig. 3d) and a dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol (Maruyama *et al.* 2000; Akao *et al.* 2002). NM may activate inner membrane anion channel through the altered redox state depending on the GSH/GSSG ratio (Aon *et al.* 2007), or the modification of vital SH in mitochondria. Diazenedicarboxylic acid bis(N,N′-dimethylamide) (diamide) induced mitochondrial permeability transition (mPT) by modifying thiol of Cys57 in adenine nucleotide translocator (ANT) localized on the matrix site at the cyclosporine-binding site (Costantini *et al.* 2000). Modification of Cys57 in ANT with NO could prevent mPT maybe by protection of the vital SH against further cytotoxic modification. EGCG, a NO scavenger, and SOD prevented the NM-induced apoptosis and increase in mitochondrial SH, suggesting the involvement of NO, superoxide, and peroxynitrite. In addition, iron released from NM may enhance ROS–RNS production, as shown by the protection of DFX. NM may remove protective GSH or cysteine from mixed disulfide bonds in mPT pores and expose vital SH to subsequent modification by ROS–RNS, resulting in induction of mPT and apoptosis, which overcomes the protection by Bcl-2.

As a conclusion, this study clearly presents data that NM induces apoptosis in SH-SY5Y cells by means of the protein component. The mechanism is clarified as that NM deglutathionylates specified PrS-SG in mitochondrial complex I, dissociates the higher structure of mitochondria, causes the dysfunctions, and finally activates apoptosis signaling. These effects of NM on the redox state were the most manifest in isolated mitochondria, where GSH is not produced *in situ* and the level is mainly regulated by GSH recycling system composed of NADPH-dependent reductase. These situations in isolated mitochondrial may reproduce those in the substantia nigra of aged and parkinsonian brains, where the redox state tends to more oxidizing condition with reduction of GSH levels. In normal condition the compensative antioxidant capacity might prevent the activation of death process by NM, even though it accumulates in the substantia nigra at the concentrations of 2–4 μg/mg wet weight, almost the same levels as used in this study, 10–25 μg/mL of reaction mixture. Further studies will clarify the more detailed role of NM in the malignant cycles between oxidative stress, mitochondrial dysfunction cell death of DA neurons in PD and aging. In addition, a quite new strategy may be found to prevent or delay the cell death itself by stabilization of mitochondrial redox state and *S*-glutathionylation.

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Formation of Dopamine Adducts Derived from Brain Polyunsaturated Fatty Acids

MECHANISM FOR PARKINSON DISEASE[*][S]

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Oxidative stress appears to be directly involved in the pathogenesis of the neurodegeneration of dopaminergic systems in Parkinson disease. In this study, we formed four dopamine modification adducts derived from docosahexaenoic acid (C22:6/ω-3) and arachidonic acid (C18:4/ω-6), which are known as the major polyunsaturated fatty acids in the brain. Upon incubation of dopamine with fatty acid hydroperoxides and an *in vivo* experiment using rat brain tissue, all four dopamine adducts were detected. Furthermore, hexanoyl dopamine (HED), an arachidonic acid-derived adduct, caused severe cytotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells, whereas the other adducts were only slightly affected. The HED-induced cell death was found to include apoptosis, which also seems to be mediated by reactive oxygen species generation and mitochondrial abnormality. Additionally, the experiments using monoamine transporter inhibitor and mouse embryonic fibroblast NIH-3T3 cells that lack the monoamine transporter indicate that the HED-induced cytotoxicity might specially occur in the neuronal cells. These data suggest that the formation of the docosahexaenoic acid- and arachidonic acid-derived dopamine adducts *in vitro* and *in vivo*, and HED, the arachidonic acid-derived dopamine modification adduct, which caused selective cytotoxicity of neuronal cells, may indicate a novel mechanism responsible for the pathogenesis in Parkinson disease.

Parkinson disease (PD)[3] is a neurodegenerative disorder characterized by a dramatic loss of dopaminergic neurons in the substantia nigra and the subsequent deficiency of dopamine in the brain areas (1). Until now, very little is known about why and how the PD neurodegenerative process begins and progresses; however, an increasing body of evidence suggests that oxidative stress, mitochondrial dysfunction, and impairment of the ubiquitin-proteasome system may be involved in the pathogenesis of PD (2–5). Recent studies indicate that there are high levels of basal oxidative stress in the substantia nigra pars compacta in the normal brain, and this is increased in PD (6).

Oxidative stress in the brain easily leads to the lipid peroxidation reaction because of a high concentration of polyunsaturated fatty acids, such as docosahexaenoic acid (DHA, C22:6/ω-3) and arachidonic acid (AA, C18:4/ω-6), which are present in the brain (7). The polyunsaturated fatty acids are located almost exclusively in the SN2 position of the phosphoglycerides found in the neural cell membranes. The beneficial physiological effects of DHA and AA have been frequently reported (8, 9); however, the fatty acids are highly unsaturated, thus making them particularly susceptible to peroxidation. During the lipid peroxidation reaction, lipid hydroperoxides are generated as primary products. Subsequent decomposition leads to the formation of reactive mediates including aldehydes, which can covalently modify biomolecules. We have recently found that lipid hydroperoxides, the primary peroxidative products, can universally react with primary amino groups to form *N*-acyl-type (amide linkage) adducts (10–15). In our previous studies, the formation of linoleic acid-derived lysine modification adducts, N^ϵ-(hexanoyl) lysine and N^ϵ-(azelayl) lysine, and DHA-derived adducts, N^ϵ-(succinyl) lysine and N^ϵ-(propanoyl) lysine, have been identified *in vitro* or *in vivo* by liquid chromatography-MS/MS or immunochemical analysis. In addition, the formation of N^ϵ-(hexanoyl) lysine also was detected, as well as N^ϵ-(glutaryl) lysine, during the reaction of oxidized AA with the lysine residue. The *N*-acyl-type adducts are specific to the peroxidation of polyunsaturated fatty acids; therefore, their formations are useful markers for the lipid peroxidation, protein modification, and related dysfunction that occur in these fatty acid-enriched tissues.

Dopamine is the endogenous neurotransmitter produced by nigral neurons. Dopamine loss can trigger not only prominent secondary morphological changes, such as density reduction of the dendritic spines, but also changes in the density and sensitivity of dopamine receptors (1); therefore, it is a sign of PD development. The reasons for dopamine loss are attributed to

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[3] The abbreviations used are: PD, Parkinson disease; DHA, docosaheaxenoic acid; AA, arachidonic acid; SUD, succinyl dopamine; PRD, propanoyl dopamine; HED, hexanoyl dopamine; GLD, glutaroyl dopamine; NOD, nonanoyl dopamine; LAD, lauroyl dopamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; 6-OHDA, 6-hydroperoxydopamine; ROS, reactive oxygen species; MS/MS, tandem mass spectrometry; HPLC, high performance liquid chromatography; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DCF, dichlorofluorescein; DAT, dopamine transporter; NET, norepinephrine transporter; 5-HTT, serotonin transporter; HEDOPA, hexanoyl dihydroxyphenylalaine.

the molecular instability of dopamine. Some possible causes of dopamine loss are abnormalities of dopaminergic neurons (16), dopamine degradation by monoamine oxidase A (17) or auto-oxidation (18) and the reaction with amino acid cysteine (19). Dopamine is a member of catecholamine family. The catechol structure contributes to high oxidative activation of dopamine. Additionally, the N termini in the structure of dopamine may represent another reactive spot; however, little experimental evidence proves this. Based on our previously described reaction between lipid hydroperoxides and N-terminal residues, we focused on the possibility that reactive hydroperoxide species derived from lipid peroxidation may modify dopamine to form amide linkage dopamine adducts.

In the present study, we chemically synthesized four dopamine-modified adducts derived from DHA and AA. We were particularly interested in the formation of the dopamine adducts by chemical reactions and *in vivo* experiments, as well as the cytotoxicity evaluation using neuronal cells. All four dopamine adducts were detected upon incubation of dopamine with fatty acid hydroperoxides and an *in vivo* experiment using rat brain tissue. Furthermore, we focused on an AA-derived adduct HED, which induced severe cytotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells compared with other adducts. The HED-induced cell death was found to include apoptosis that might be mediated by reactive oxygen species (ROS) and mitochondrial abnormality in SH-SY5Y cells. In addition, we found that the presence of monoamine transporters in the cells was essential for the HED-induced cytotoxicity, suggesting the specificity of the cytotoxicity to the cells. Taken together, the DHA- and AA-derived dopamine adducts may be useful biomarkers of the dopamine deficiency, and the formation of these adducts may indicate a novel mechanism responsible for the pathogenesis in Parkinson disease.

EXPERIMENTAL PROCEDURES

Materials—DHA, arachidonic acid, lipoxidase, GBR 12909 dihydrochloride, imipramine hydrochloride, *N*-acetyl-Asp-Glu-Aal-Asp-al, and RNase were obtained from Sigma-Aldrich (Tokyo, Japan). Dopamine·HCl was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Linoleic acid, hexanoic anhydride, glutaric anhydride, propanoic anhydride, succinic anhydride, lauric anhydride, and nonanoic anhydride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The antibodies against cytochrome *c* oxidase IV and poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology, Inc. (Boston, MA). Active caspase-3 rabbit monoclonal antibody was purchased from Epitomics, Inc. (Burlingane, CA).

Synthesis of N-Acyl Dopamine Adducts—The *N*-acyl dopamine adducts were chemically synthesized by incubating dopamine (0.5 mM) with carboxylic or methylic anhydride (0.5 mM) in 5 ml of 100 mM sodium phosphate buffer (pH 7.4)-saturated sodium acetate (1:1, v/v) for 60 min at room temperature. Succinic anhydride and propanoic anhydride were utilized for preparing the succinyl dopamine (SUD) and propanoyl dopamine (PRD), respectively; Hexanoic anhydride and glutaric anhydride were used for synthesizing the HED and glutaroyl dopamine (GLD), respectively. The synthesized adducts were purified by reverse phase HPLC using a Develosil ODS-HG-5 column (20 × 250 mm) in an isocratic system of 15 or 50% acetonitrile containing 0.1% trifluoroacetic acid at the flow rate of 6 ml/min. The elution profiles were monitored by absorbance at 280 nm. The amino residues in the dopamine adducts were identified by the ninhydrin reaction. The mass, structure, and formula of the synthesized molecule were identified by HPLC-MS, NMR, and electrospray ionization time-of-flight mass spectrometry analyses, respectively.

HPLC-Tandem Mass Spectrometry—The HPLC-MS/MS analysis was carried out using an API 2000 triple quadrupole mass spectrometer (Applied Biosystems) through a TurboIon-Spray source. Chromatography was carried out on a Develosil ODS-HG-3 column (2.0 × 250 mm) using an Agilent 1100 HPLC system. The chromatographic separation was performed by a gradient elution as follows: 0–10 min, linear gradient from 0.1% formic acid to 50% aqueous acetonitrile containing 0.1% formic acid; 10–15 min, hold; 15–20 min, linear gradient to 0.1% formic acid; flow rate = 0.2 ml/min. The instrument response was optimized by infusion experiments with the standard compounds using a syringe pump at the flow rate of 5 μl/min. The dopamine adducts were detected using electrospray ionization MS/MS in the multiple reaction monitoring mode.

In Vitro Modification of Dopamine—DHA hydroperoxides were prepared from the DHA auto-oxidative reaction, and AA hydroperoxides were prepared using 15-lipoxygenase. The levels of lipid hydroperoxide were determined using a lipid hydroperoxide assay kit (Cayman Chemical Co., Ann Arbor, Michigan). Dopamine (2 mM) was incubated with 10 mM of DHA or AA hydroperoxides in phosphate buffer (pH 7.4) at 37 °C for different times. The reaction was terminated by immediate freezing at −80 °C. AA hydroperoxide was prepared as described previously (11).

In Vivo Detection of Dopamine Adducts—Brain homogenates of 7- and 27-week-old male F344/NSlc rats were used to detect the dopamine adducts. Briefly, the rat brain was removed and homogenized with phosphate-buffered saline containing 5% dibutylhydroxytoluene (5 mM) and EDTA (250 mM). After the addition of the deuterated dopamine adducts (20 μM) as the internal standard, the homogenates were centrifuged at 3,000 rpm for 10 min. The pellet was then dissolved in 100 μl of methanol. The detection was carried out by HPLC-MS/MS.

Cell Cultures and Drug Treatment Procedures—SH-SY5Y human dopaminergic neuroblastoma cells and NIH-3T3 mouse embryonic fibroblast cells were kindly donated by Dr. Maruyama (National Institute for Longevity Science). SH-SY5Y cells and NIH-3T3 cells were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% FBS and DMEM containing 10% FBS, respectively, and maintained at 37 °C in an atmosphere of 5% CO_2 in air. The 80% confluent cells were allowed to medium change with FBS-free DMEM overnight. The dissolved drugs were diluted by 1/500 or 1/1000 and added to fresh FBS-free DMEM to achieve the required concentration.

Assessment of Cell Viability—Cell viability was evaluated by an MTT assay. SH-SY5Y cells in 96-well plates were incubated with drugs for different times, followed by further incubation with 500 μg/ml MTT at 37 °C for 2 h. Cell viability in some experiments was also measured using PI and Hoechst 33258 staining.

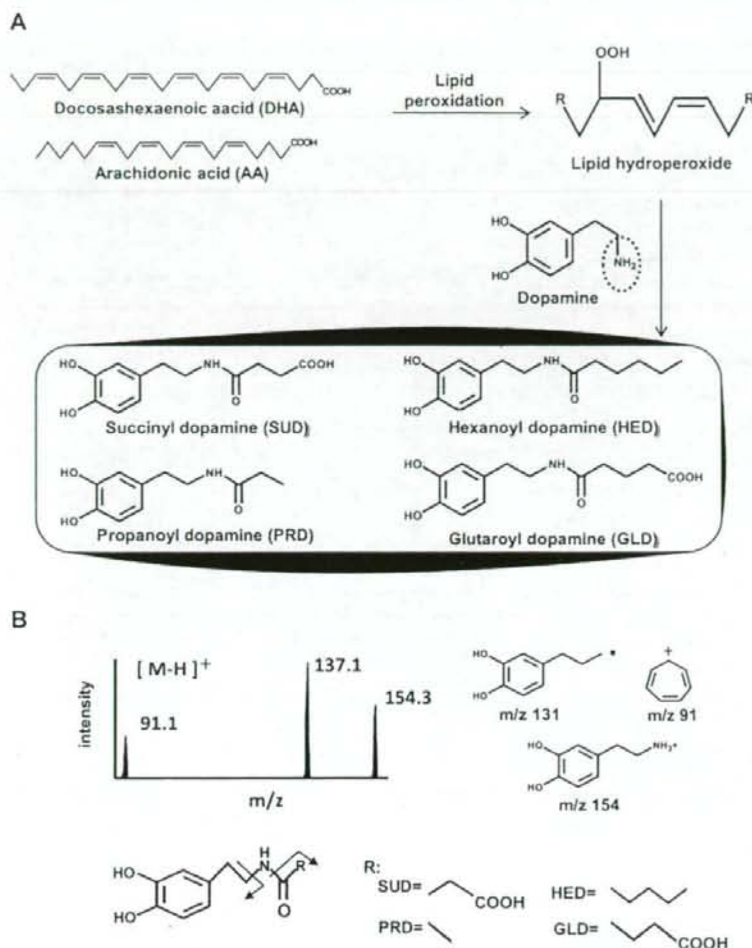

FIGURE 1. **Proposed chemical formation scheme and HPLC-MS/MS analysis of DHA- and AA-derived dopamine adducts.** *A*, proposed reaction scheme of DHA- and AA-derived dopamine adduct formation. *B*, the $[MH]^+$ ion *m/z* 254, 210, 252, and 268 of SUD, PRD, HED, and GLD, respectively, were subjected to collision-induced dissociation, and the daughter ions were scanned (*upper left panel*). The proposed structures of individual ions are shown (*upper right panel*). The chemical structure composition of the dopamine adducts is proposed by fragmental analysis (*lower panel*).

then separated on an ethidium bromide (0.5 mg/ml)-containing 2% agarose gel.

Subcellular Fraction of SH-SY5Y Cell—The cells are harvested by centrifugation at 600 × *g* for 10 min, washed with phosphate-buffered saline, and resuspended with 5 volumes of Solution A (0.25 M sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The cellular suspension was homogenized with a glass-glass homogenizer with 20 up and down passes of the pestle. The homogenate was then centrifuged at 750 × *g* for 10 min. The resulting supernatant was collected and then centrifuged at 10,000 × *g* for 15 min. The pellet was used as the mitochondrial fraction.

Western Blot Analysis—The cells were washed twice with phosphate-buffered saline, pH 7.0, and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride). After protein quantification, equal amounts of the protein (total protein, 20–50 μg) were boiled with Laemmli sample buffer for 5 min at 100 °C. The samples were run on 10% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, incubated with 5% skim milk in TTBS (Tris-buffered saline containing 10% Tween 20) for blocking, washed, and treated with the primary antibodies. After washing with TTBS, the blots were further incubated for 1 h at room temperature with the IgG antibody coupled to horseradish peroxidase in TTBS. The blots were then washed three times in TTBS before visualization. An ECL kit was used for detection.

ROS Measurement—Endogenous ROS level was detected by flow cytometry using H_2DCF-DA (2′,7′,-dichlorodihydrofluorescein diacetate) (Molecular Probes). Briefly, the drug-treated cells were incubated with H_2DCF-DA for 30 min, and the fluorescence of dichlorofluorescein (DCF) was measured using an EPICS Elite Flow Cytometer.

DNA Fragmentation Assay—The drug-treated SH-SY5Y cells were collected, suspended in 0.2 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 0.5% Triton X-100), and incubated at room temperature for 10 min. The samples were then centrifuged at 12,000 × *g* for 10 min, and the supernatant containing the DNA cleavage products was incubated with 0.2 mg/ml proteinase K at 37 °C for 1 h followed by 0.1 mg/ml RNase A for 30 min at 50 °C. The DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation and

Statistical Analysis—All of the data were analyzed using Bonferroni/Dunn's multiple comparison procedure.

RESULTS

Chemical Formation of DHA- and AA-derived Dopamine Adducts—Based on the observations that lipid hydroperoxides, the primary products of lipid peroxidation, could universally react with primary amino groups to form *N*-acyl-type (amide linkage) adducts, and also within the chemical structure of dopamine, an amino residue is present, we chemically synthesized the four amide linkage dopamine adducts, SUD, PRD, HED,

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/NSlc 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 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.

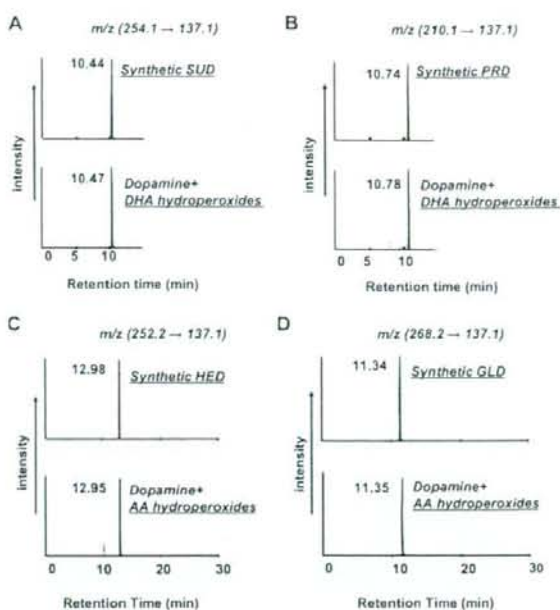

FIGURE 2. **HPLC-MS/MS analysis of the dopamine adducts formed during the reaction of dopamine with oxidized DHA and AA hydroperoxides.** Dopamine (2 mM) was incubated with lipid hydroperoxides (10 mM) in 0.1 M phosphate buffer (pH 7.4) at 37 °C. Shown is selected ion monitoring of the transitions from *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.

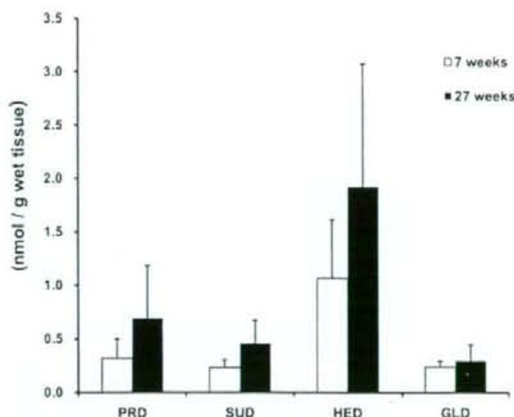

FIGURE 3. **Formation of the dopamine adducts *in vivo*.** The levels of dopamine adduct formed in rat brain were determined by HPLC-MS/MS (data are shown as the means ± S.D. (n = 5)).

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

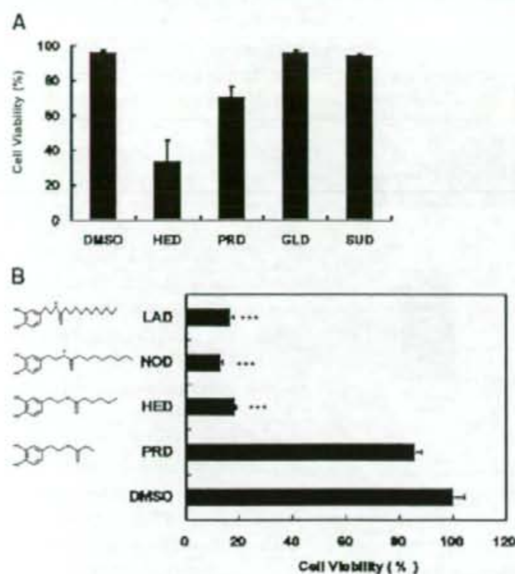

FIGURE 4. **Identification of HED as a potent inducer of cell death in SH-SY5Y cells.** The cells were exposed to 100 μM sample for 24 h. Cell viability was measured by the MTT assay. In the MTT assay, the data are expressed as percentages of control culture conditions. *A*, potential comparison of cell 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

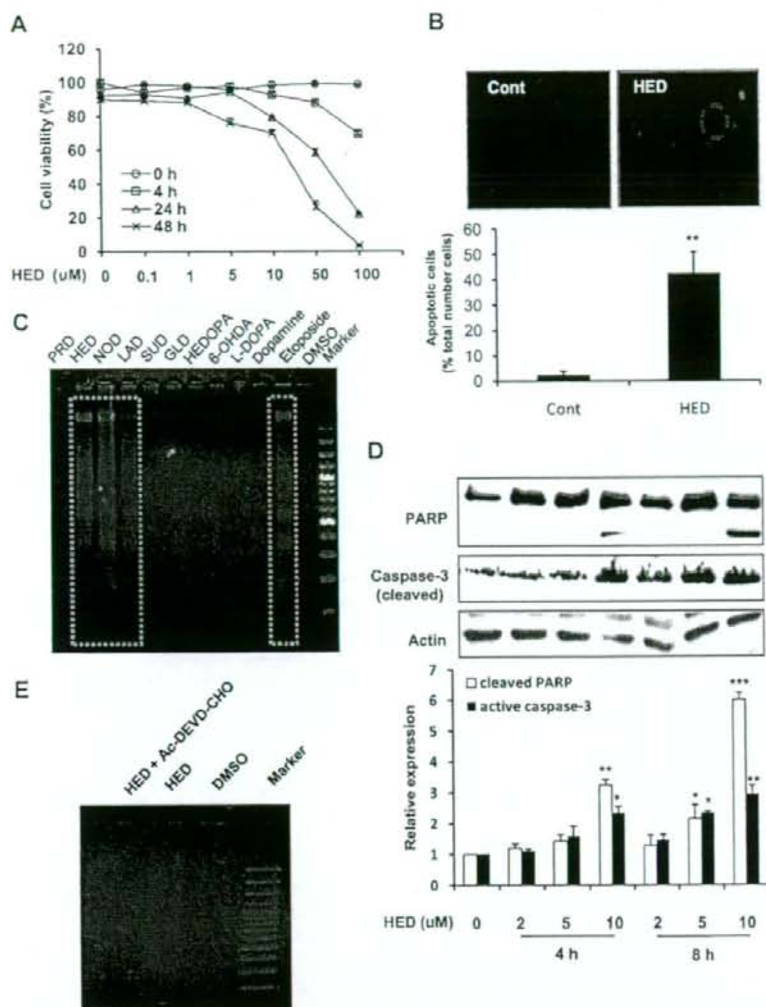

FIGURE 5. **Apoptosis induced by HED.** *A*, dose- and time-dependent cytotoxicity of HED. SH-SY5Y cells were exposed to 0–100 μM HED for different retention times. Cell viability was measured by the MTT assay. *B*, chromatin condensation in SH-SY5Y cells exposed to 10 μM HED. The cells were fixed with 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

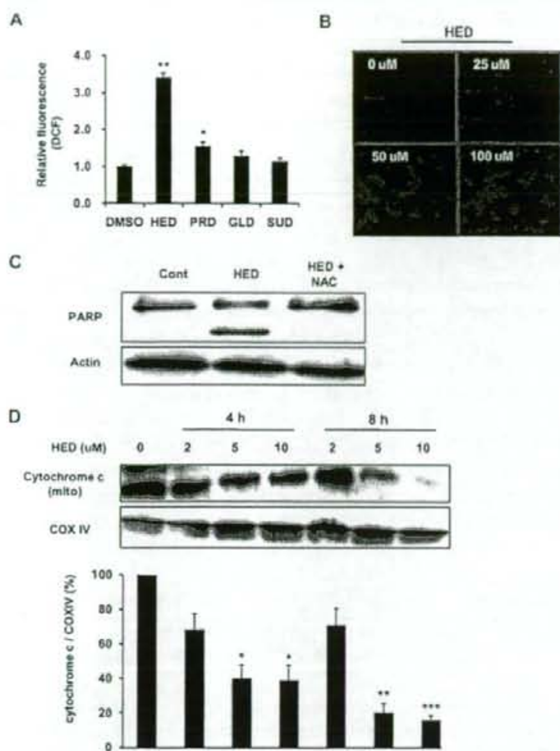

FIGURE 6. **ROS generation and cytochrome *c* release during HED-induced apoptosis.** *A*, comparison of ROS generation inducted by DHA- and AA-derived dopamine adducts. The SH-SY5Y cells were treated with 10 μM dopamine adducts for 30 min and exposed to H_2DCF-DA for 30 min. The fluorescence of DCF 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.

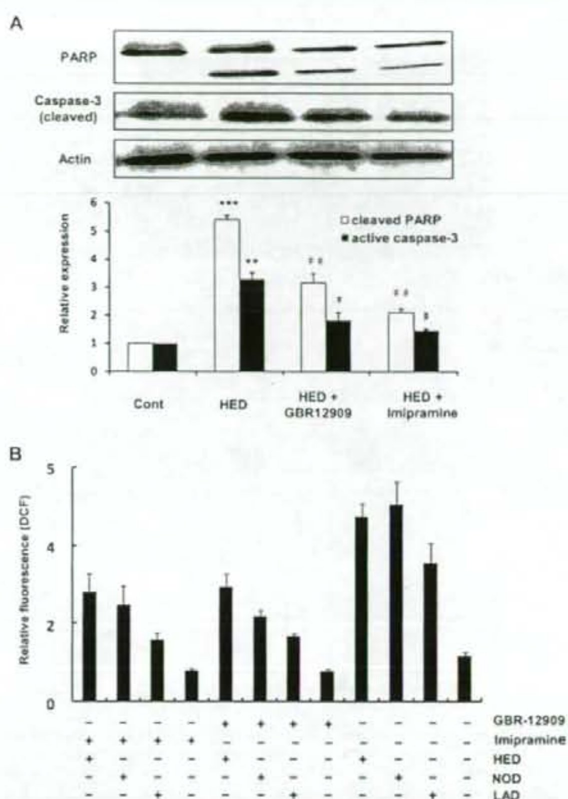

FIGURE 7. **Effect of monoamine transporter inhibitors on apoptosis and ROS generation.** The inhibitors used were GBR 12909 and imipramine for DAT and NET/5-HTT, respectively. 1 μM inhibitors were administrated in SH-SY5Y cells for 30 min before drug treatments. *A*, effect of monoamine transporter 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

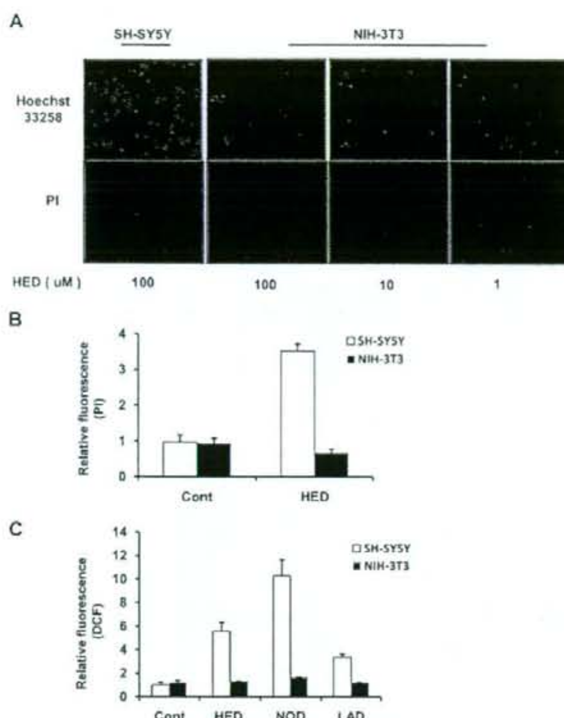

FIGURE 8. **No cytotoxicity was induced by HED in NIH3T3 cells compared with in SH-SY5Y cells.** *A*, apoptotic cells imaging. NIH3T3 cells were treated with different concentrations of HED for 12 h. PI and Hoechst staining were performed by fluorescence microscope. *B*, numbers of apoptotic cells. The 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 $\approx$ NOD $\approx$ 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

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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

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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.

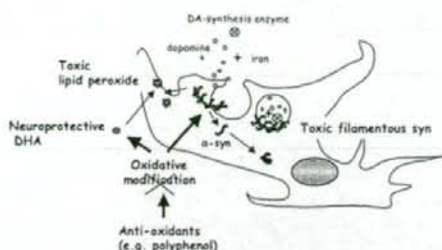

Fig. 1. DHA is generally neuroprotective, but potentially neurotoxic when the oxidative stress is increased. Under the oxidative stress, DHA may produce toxic lipid peroxide and produce toxic protein adducts. Polyphenol or other food-derived antioxidants might prevent the toxicity of DHA by reducing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

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keyword
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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