Oxidative Stress Induces Intracellular Accumulation of Amyloid β-Protein (Aβ) in Human Neuroblastoma Cells

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ABSTRACT: Several lines of evidence suggest that enhanced oxidative stress is involved in the pathogenesis and/or progression of Alzheimer's disease (AD). Amyloid β-protein (Aβ) that composes senile plaques, a major neuropathological hallmark of AD, is considered to have a causal role in AD. Thus, we have studied the effect of oxidative stress on Aβ metabolism within the cell. Here, we report that oxidative stress induced by H_2O_2 (100−250 μM) caused an increase in the levels of intracellular Aβ in human neuroblastoma SH-SY5Y cells. Treatment with 200 μM H_2O_2 caused significant decreases in the protein levels of full-length β-amyloid precursor protein (APP) and its COOH-terminal fragment that is generated by β-cleavage, while the gene expression of APP was not altered under these conditions. A pulse-chase experiment further showed a decrease in the half-life of this amyloidogenic COOH-terminal fragment but not in that of nonamyloidogenic counterpart in the H_2O_2-treated cells. These results suggest that oxidative stress promotes intracellular accumulation of Aβ through enhancing the amyloidogenic pathway.

Oxidative stress has long been implicated in a number of age-associated disorders, neurodegenerative diseases, including Alzheimer's disease (AD),[1] and finally in aging itself (*1*). Oxidative stress is defined as the conditions under which free radicals in excess of the antioxidant defense mechanisms are present. Within the cell, H_2O_2 is produced nonenzymatically or by several oxidases during normal metabolism and provides a source of hydroxyl radicals (•OH) which are highly reactive and capable of oxidizing lipids, carbohydrates, proteins, and DNA. It is thus an important pro-oxidant that produces oxidative stress in vivo. Together with H_2O_2, singlet oxygen, and hypochloric acid, oxygen free radicals are often called reactive oxygen species (ROS) (*1*, *2*).

With aging, increased formation of ROS and the consequent membrane alterations are observed even in normal brains and may be even more marked in AD brains (*2*). Thus, oxidative stress may be involved in the pathogenesis and/or progression, if not the etiology, of AD. There is now considerable support for this assumption. The levels of several molecular species representing in vivo oxidative stress were found to be elevated in the AD brain, including 4-hydroxynonenal (*3*), isoprostanes (*4*, *5*), 8-hydroxy-2′-guanosine and -deoxyguanosine (*6*, *7*), protein carbonyls (*8*), and nitrotyrosine (*9*). The first two represent lipid peroxidation, the third, RNA and DNA oxidation, and the last two, protein oxidation. On the other hand, increased expression and protein content of heme oxygenase 1, an antioxidant enzyme induced by oxidative stress, has been repeatedly reported (*10*, *11*). Although the source of ROS is not yet confirmed, one strong possibility is the mitochondria, which continuously generate ATP through reduction of O_2 by sequential addition of electrons and H^+ (*1*). Another possibility is the redox-active Fe which was reported to be associated with neurofibrillary tangles and senile plaques in the AD brain (*12*). A hydroxyl radical is stoichiometrically generated by oxidation of iron(II) by H_2O_2 to iron(III), which is called the Fenton reaction. All these findings suggest that cumulative oxidative stress contributes to the progression and/or pathogenesis of AD, an age-dependent neurodegenerative disease.

It was reported that glycated τ induced oxidative stress thereby activating transcription via NFκB, which results in increased levels of β-amyloid precursor protein (APP) and amyloid β-protein (Aβ) secretion from neuroblastoma cells (*13*). This raises the possibility that oxidative stress increases the level of intracellular Aβ and causes a greater extent of Aβ secretion into the extracellular space and thus may contribute to β-amyloid deposition. In fact, previous reports described the alterations elicited by H_2O_2 in the processing of APP and levels of Aβ in lens epithelial cells (*14*) and during H_2O_2-induced apoptosis in human neuroblastoma cells

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[1] Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; APP, β-amyloid precursor protein; C83, the fragment containing 83 amino acids of the COOH-terminus of APP; C99, the fragment containing 99 amino acids of the COOH-terminus of APP; CHO cells, Chinese hamster ovary cells; DIG, detergent-insoluble, glycolipid-enriched domain; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ERAB/HADH, ER-associated Aβ-binding protein/hydroxyacyl-CoA dehydrogenase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

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(*15*). However, work neither unambiguously quantitated the levels of intracellular Aβ nor distinguished between Aβ42 and Aβ40.

In addition, a significant fraction of intracellular Aβ exists in a distinct membrane compartment which is characterized by its insolubility in nonionic detergents (*16*−*18*). Thus, the Aβ in the detergent-insoluble as well as detergent-soluble compartment should be quantitated to learn how oxidative stress affects intracellular Aβ metabolism. In the present study on nontransfected human neuroblastoma SH-SY5Y cells, we investigated the effects of H_2O_2 on the levels of Aβ in these compartments using a sensitive enzyme-linked immunosorbent assay (ELISA), and its effects on the processing of APP and its COOH-terminal fragments using Western blotting and metabolic labeling.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents. The antibodies used for the sandwich ELISA were BAN50 (raised against Aβ 1−16), BA27 (highly specific for Aβ40), and BC05 (specific for Aβ42) (*19*). The antibodies, 4G8 (specific for Aβ 17−24) and 6E10 (raised against Aβ 1−17), were purchased from Senetek PLC (Maryland, MO). Anti-APP antiserum C4 was raised against a synthetic peptide of APP 666−695 (according to the numbering of APP695) which corresponds to the cytoplasmic domain of APP (*17*). Anti α-tubulin antibody was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and Trolox from Calbiochem-Novabiochem (San Diego, CA).

Cell Culture. SH-SY5Y cells (a gift from Dr. C. L. Masters, University of Melbourne, Australia) were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (1:1 mixture) containing nonessential amino acids (Eagle's formulation) and 10% fetal bovine serum (FBS). Cells were plated onto culture dishes coated with collagen type I. Chinese hamster ovary cells stably transfected with APP751 (CHO-APP751 cells; a gift from Dr. E. H. Koo, University of California) were cultured in DMEM supplemented with 10% FBS (*20*).

Assays for Cytotoxicity and Apoptosis. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as previously described (*21*), with minor modifications. Briefly, cells were incubated with H_2O_2 in the culture medium for 24 h at 37 °C and then with 50 μg/mL MTT for another 1 h. Absorption at 570 nm was measured after solubilization of the formazan crystals with 0.04 M HCl in 2-propanol. The MTT-reducing activity was expressed as a percentage of the control.

To detect apoptotic nuclei, H_2O_2-treated cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with the fluorescent DNA-binding dye, Hoechst 33258 (Molecular Probes, Eugene, OR). The number of apoptotic condensed nuclei was counted twice in the same field (three random fields containing 200 cells) and their extent was expressed as a percentage relative to the number of total nuclei. Alternatively, translocated phosphatidylserine on the cell surface was detected with fluorescein isothiocyanate (FITC)-conjugated annexin V (Sigma, St. Louis, MO) (*22*). To this end, H_2O_2-treated cells were incubated with 1 μg/mL FITC-annexin V in 140 mM NaCl, 2.5 mM $CaCl_2$, and 10 mM Hepes (pH7.4) at room temperature for 20 min. The stained cells were photographed using a 40 x objective.

Subcellular Fractionation and Quantitation of Aβ. Semiconfluent cells were incubated for 24 h in media containing various concentrations of H_2O_2. After being washed with phosphate-buffered saline (PBS), the cells were collected by centrifugation and homogenized on ice with a glass/Teflon homogenizer in 5 vol of 1% Triton X-100 in 150 mM NaCl and 50 mM Tris-HCl (pH 7.6). The homogenate was centrifuged at 540000*g* for 20 min in a TLX ultracentrifuge (Beckman, Palo Alto, CA), and the supernatant (Triton-soluble fraction) was subjected to ELISA. The pellet was homogenized and centrifuged again to wash away any remaining detergent-soluble proteins. It was then suspended by brief sonication in an equal volume (with respect to the initial volume) of 6 M guanidine hydrochloride in 50 mM Tris-HCl (pH 7.6). The resulting supernatant following a centrifugation at 265000*g* for 20 min was diluted 1:12 and subjected to ELISA to quantitate the levels of Aβ in the Triton-insoluble fraction. ELISA was performed as previously described (*17*). ELISA plates were coated with BAN50, and the captured Aβ was detected using BA27 or BC05 monoclonal antibody conjugated with horseradish peroxidase. The levels of Aβ were expressed as femtomoles per milligram of protein or as a percentage of the level in the control.

Immunoprecipitation. Triton-insoluble fractions were first dialyzed against 0.5 M guanidine hydrochloride in 50 mM Tris-HCl (pH 7.6). Both Triton-soluble and -insoluble fractions were incubated overnight at 4 °C with BAN50 (15 μg) to immunoprecipitate Aβ, or antiserum C4 (1:500) to immunoprecipitate the COOH-terminal fragments of APP. The resultant immunocomplexes were collected by incubation with 2.5 μL of protein G-Sepharose 4FF (Amersham Pharmacia Biotech) for 2 h. The bound proteins were eluted with the Laemmli sample buffer (2% SDS, 10% glycerol, 2.5% 2-mercaptoethanol, and 50 mM Tris-HCl, pH 6.8) and subjected to Western blotting.

Western Blotting. Proteins separated on an SDS−polyacrylamide gel were transferred to a poly(vinylidene difluoride) membrane (Millipore, Bedford, MA). The bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). For the detection of Aβ, proteins were separated on 16.5% Tris/Tricine gels and transferred to a nitrocellulose membrane (Schleicher & Schnell, Keene, NH) as previously described (*17*). The membrane was boiled in PBS for 5 min to enhance the sensitivity (*23*). The intensity of the visualized bands was quantitated using a model GS-700 imaging densitometer on Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA).

Pulse-Chase Experiment. Cells treated with 200 μM H_2O_2 were incubated with methionine-free DMEM supplemented with 10% dialyzed FBS for 60 min. The cells were metabolically labeled with 300 μCi/mL [^{35}S]methionine for 60 min and then chased with medium containing methionine for 30, 60, and 120 min at 37 °C. After the cells were washed with ice-cold PBS, they were collected by centrifugation and lysed with 10 vol of 1% Triton X-100 for 30 min on ice. The lysate was centrifuged at 18500*g* for 15 min, and the supernatant was incubated overnight at 4 °C with antiserum C4 (1:200). The resulting immunocomplexes were collected with protein G-Sepharose 4FF as described above. The bound proteins were eluted with the sample buffer and subjected

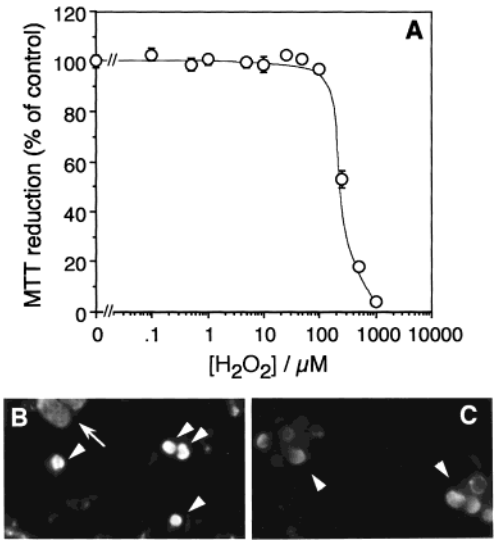

FIGURE 1: H_2O_2 induced apoptosis of SH-SY5Y cells. (A) SH-SY5Y cells were treated with H_2O_2 for 24 h at 37 °C, and MTT reduction was determined by absorption at 570 nm. Values are expressed as percentages (means ± sem; $n = 4$) of the values in untreated controls. Error bars within symbols are omitted. (B) SH-SY5Y cells were treated with 200 μM H_2O_2 for 24 h at 37 °C and fixed with 4% paraformaldehyde. Nuclei were stained with 1 μg/mL Hoechst 33258. The arrow indicates the nucleus of a viable cell, while arrowheads indicate the condensed nuclei of apoptotic cells (39 ± 6% of total). (C) Cells similarly treated with 200 μM H_2O_2 for 24 h were stained with FITC-annexin V for translocated phosphatidylserine. Arrowheads indicate apoptotic cells. Viable cells were not stained with FITC-annexin V.

to electrophoresis on 16.5% Tris/Tricine gel. The radioactive bands were visualized and quantitated using Fujix Bio-Imaging Analyzer (model 2000, Fuji Photo Film, Tokyo, Japan). Regression analysis was performed using StatView software (version 5, SAS Institute Inc., Cary, North Carolina).

Northern Blot Hybridization. Ten micrograms of total RNA was separated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and cross-linked to the membrane by UV irradiation. The blot was hybridized overnight with cDNA probes labeled with [^{32}P]dCTP by random priming extension; the cDNA probes used were human APP695 full-length cDNA (2.1 kb) and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA (Clontech, Heidelberg, Germany). Hybridized probes were visualized and quantitated using Fujix Bio-Imaging Analyzer.

RESULTS

H_2O_2-Induced Apoptosis of SH-SY5Y Cells. H_2O_2 can freely penetrate the cell membrane and generates the highly reactive hydroxyl radical, ·OH, within the cell. It was reported that H_2O_2 induces apoptosis in various types of cells, including SH-SY5Y cells (*15*). To determine the effective concentrations of H_2O_2, SH-SY5Y cells were treated with various concentrations (100 nM to 1 mM) of H_2O_2 for 24 h, and the viability of the cells was assessed by the MTT-reducing activity. Treatment with 100 μM H_2O_2 or less had virtually no effect on the viability of SH-SY5Y cells under the employed conditions, while with the concentrations higher than 100 μM, the extent of MTT reduction decreased steeply (Figure 1A). The MTT-reducing activity decreased to 48% of that in untreated cells with 200 μM H_2O_2, and the activity was almost abolished with 1 mM H_2O_2 (Figure 1A). To characterize the cell death induced by H_2O_2, the treated SH-SY5Y cells were stained with Hoechst 33258 and with FITC-labeled annexin V to see phosphatidylserine on the cell surface: during apoptosis, phosphatidylserine translocates from the cytoplasmic leaflet to the outer leaflet of the plasma membrane (*22*). In SH-SY5Y cells treated with 200 μM H_2O_2, condensed nuclei (Figure 1B) and increased surface phosphatidylserine (Figure 1C) were observed. Approximately 39 ± 6% of total nuclei showed condensed form in the presence of 200 μM H_2O_2, whereas only 6 ± 1% of the nuclei were abnormal in untreated control. The discrepancy between the results obtained from MTT assay and those from Hoechst staining might be due to loss of dead cells during wash and fixation for Hoechst staining. In any event, these results indicate that H_2O_2 at this concentration induced apoptosis of SH-SY5Y cells.

H_2O_2 Induced an Accumulation of Intracellular Aβ in SH-SY5Y Cells. We and others previously reported that Aβ is detectable in the nonionic detergent-insoluble fraction as well as in the detergent-soluble fraction of cells (*16*−*18*). Thus, we investigated the effects of H_2O_2 on the levels of intracellular Aβ in the Triton X-100-soluble and -insoluble fractions separately. The levels of Aβ in each fraction were quantitated using BAN50-based ELISA, and thus values most likely representing Aβ1−40 and Aβ1−42 were obtained. Aβ40 and Aβ42 were detected in both Triton-soluble and -insoluble fractions, and the levels of Aβ42 in the Triton-insoluble fractions were higher than those in the Triton-soluble fractions (Figure 2), an observation which is consistent with that previously reported (*17*). The levels of Aβ40 in the Triton-insoluble fraction in the present study (∼0.4 fmol/mg protein) were lower than those reported in a previous study (∼0.9 fmol/mg protein). Under the culture conditions employed, we were unable to detect secreted Aβ40 or Aβ42 in the culture media, either by ELISA or by immunoprecipitation (data not shown); this was a highly reproducible observation. This suggests that our SH-SY5Y cells may have lost the capability of secreting detectable amounts of Aβ into the culture medium (see Discussion).

We next examined the effect of H_2O_2 on the levels of Aβ in the two fractions. Even when the cells were treated with 100 μM H_2O_2, a concentration which scarcely affected the cell viability (Figure 1A), the levels of Aβ40 in the Triton-soluble fraction were significantly increased (Figure 2A). At the concentrations of 200 and 250 μM, which induced apoptosis of 50−70% of the cells (Figure 1A), a 2−3-fold increase in the levels of Aβ40 in the Triton-soluble fraction was observed (Figure 2A). The same concentrations of H_2O_2 also caused a 2-fold increase in the levels of Aβ40 in the Triton-insoluble fraction (Figure 2B). In contrast to Aβ40, the levels of Aβ42 in the Triton-soluble fraction showed only a smaller, 40−70%, increase at 200 and 250 μM of H_2O_2 (Figure 2A), and its levels in the Triton-insoluble fraction were only slightly (but not statistically significant) increased even at 250 μM H_2O_2 (Figure 2B). The increase of Aβ in both fractions induced with 200 μM H_2O_2 was blocked by 200 μM Trolox, a water-soluble derivative of vitamin E (Table 1). Trolox also suppressed the cytotoxic effect of H_2O_2: percentages of MTT-reducing activities compared to untreated control cells were 58.64 ± 1.82 (mean ± sem) for

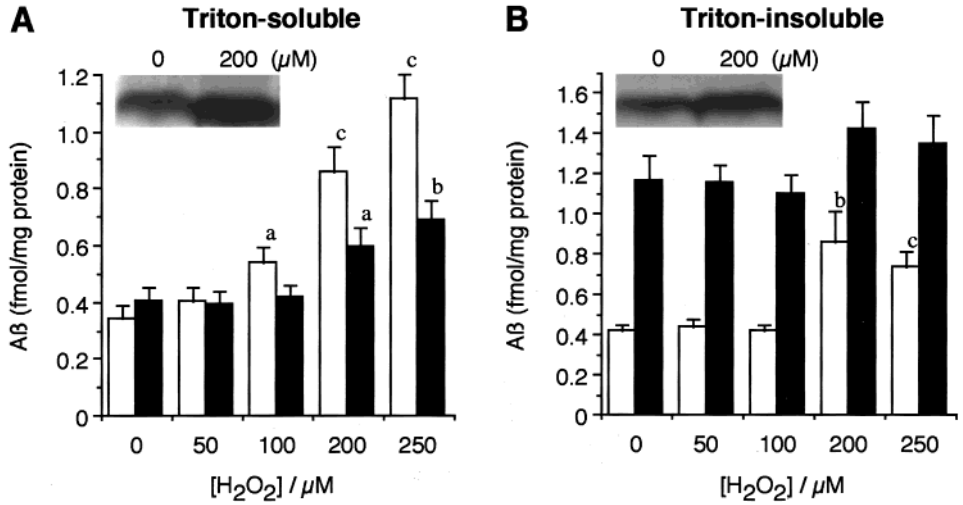

FIGURE 2: Effects of H_2O_2 on the levels of intracellular Aβ in Triton-soluble and -insoluble fractions. SH-SY5Y cells were treated with the indicated concentrations of H_2O_2 for 24 h at 37 °C, and homogenized in 1% Triton X-100. After ultracentrifugation, the insoluble residue was extracted with 6 M guanidine hydrochloride. The levels of Aβ40 (open column) and Aβ42 (closed column) in the Triton-soluble (A) and -insoluble (B) fractions were quantitated using ELISA, and are expressed as femtomoles per milligram of protein (means ± sem; $n = 10$). Statistical significance is indicated as *a*, $p < 0.05$; *b*, $p < 0.01$; *c*, $p < 0.001$. (Inset) The cells were treated with 200 μM H_2O_2 for 24 h at 37 °C. Aβ in the Triton-soluble and -insoluble fractions was immunoprecipitated with BAN50, and subjected to Western blotting with 6E10.

Table 1: Effect of Trolox on H_2O_2-Induced Aβ Accumulation[a]

	levels of Aβ (% of control)[b]			
	Triton-soluble		Triton-insoluble	
	Aβ40	Aβ42	Aβ40	Aβ42
control	100.00 ± 3.03	100.00 ± 7.00	100.00 ± 4.26	100.00 ± 3.55
H_2O_2 200 μM[c]	248.17 ± 24.34	167.73 ± 28.16	233.25 ± 38.77	148.84 ± 10.37
Trolox 200 μM	116.07 ± 10.58	74.04 ± 5.30	91.86 ± 3.41	95.28 ± 7.28
Trolox + H_2O_2[d]	119.00 ± 14.91	88.95 ± 7.08	96.92 ± 4.83	104.62 ± 9.81

[a] SH-SY5Y cells were treated with 200 μM H_2O_2 for 24 h at 37 °C in the presence or absence of 200 μM Trolox, and the levels of Aβ were measured using ELISA. [b] The values are expressed as percentages of those in untreated cells. Data are shown as means ± sem ($n = 4$). [c] $p < 0.001$, compared to control. [d] $p < 0.005$, compared to H_2O_2.

H_2O_2-treated cells and 94.32 ± 4.55 for the cells treated with both H_2O_2 and Trolox. Trolox itself reduced the level of Aβ42 in the Triton-soluble fraction for some unknown reason.

To further confirm the increase in the Aβ levels, these fractions were immunoprecipitated with BAN50 and the immunocomplexes were subjected to Western blotting using 6E10. As shown in each inset in Figure 2, the signal intensity at 4 kDa was obviously increased in the both fractions from the cells treated with 200 μM H_2O_2. Although we were unable to distinguish between Aβ40 and Aβ42 by Western blotting, the results appeared to be consistent with the results obtained by ELISA. These results strongly suggest that H_2O_2 induces an accumulation of intracellular Aβ both in the Triton-soluble and -insoluble fractions of SH-SY5Y cells.

Aβ Accumulation in CHO Cells Stably Transfected with APP751. To see whether H_2O_2 similarly induces accumulation of Aβ in other cells, we used CHO-K1 cells stably transfected with APP751 (*20*). CHO-APP751 cells were treated with 800 μM H_2O_2 at which concentration 40% of cells became apoptotic (data not shown). Under these conditions, the levels of Aβ in the Triton-soluble and -insoluble fractions of the CHO cells were quantitated by ELISA. The levels of Aβ were 10-fold higher than those in SH-SY5Y cells, and Aβ40 was the predominant species in both the Triton-soluble and -insoluble fractions. Thus, as

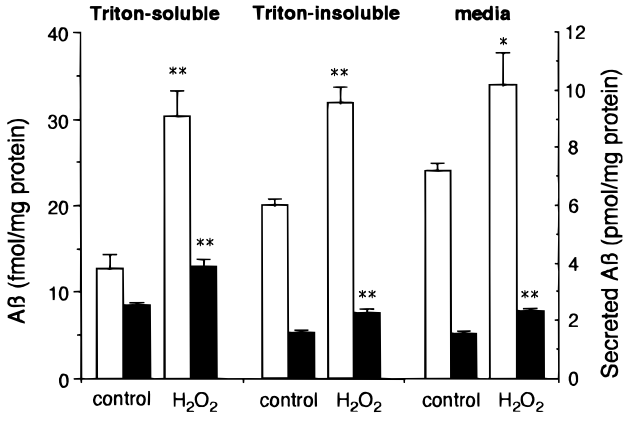

FIGURE 3: Effects of H_2O_2 on the levels of intracellular Aβ in CHO cells stably transfected with APP751. CHO cells transfected with APP751 were treated with 800 μM H_2O_2 for 24 h at 37 °C and homogenized in 1% Triton X-100. After ultracentrifugation, the insoluble residue was extracted with 6 M guanidine hydrochloride. The levels of Aβ40 (open column) and Aβ42 (closed column) in the Triton-soluble and -insoluble fractions, and the amount of Aβ secreted for 24 h were quantitated using ELISA. The levels in the Triton-soluble and -insoluble fractions are expressed as femtomoles per milligram of protein, and the levels of secreted Aβ as picomoles per milligram of protein (means ± sem). *$p < 0.05$; **$p < 0.005$.

shown in Figure 3, H_2O_2 also caused increases in the levels of Aβ40 and Aβ42 in both the fractions of CHO-APP751

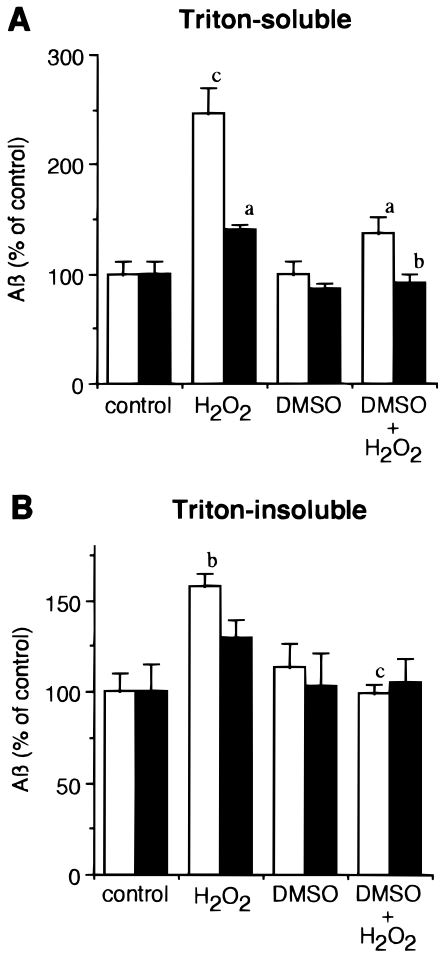

FIGURE 4: Effect of dimethyl sulfoxide (DMSO), a scavenger of hydroxyl radicals, on H_2O_2-induced accumulation of Aβ. SH-SY5Y cells were treated with 200 μM H_2O_2 for 24 h at 37 °C in the presence or absence of 0.1% DMSO. The levels of Aβ40 (open column) and Aβ42 (closed column) in the Triton-soluble (A) and -insoluble (B) fractions were determined using ELISA. Values are expressed as percentages of the Aβ levels in untreated cells. Data are the means ± sem (n =6). Statistical significance is indicated as *a*, $p < 0.01$; *b*, $p < 0.001$; *c*, $p < 0.0001$. Control vs H_2O_2, and H_2O_2 vs DMSO + H_2O_2 were compared.

cells. The levels of Aβ in the Triton-soluble fraction were more sensitive to H_2O_2 than those in the Triton-insoluble fraction, which is similar to the results in SH-SY5Y cells. Notably, in CHO-APP751 cells, H_2O_2 treatment enhanced the secretion of both Aβ40 and Aβ42. These results indicate that the intracellular accumulation of Aβ induced by H_2O_2 is not a particular event specific for only SH-SY5Y cells that do not secrete Aβ and suggest that interference with Aβ secretion is not the cause of intracellular Aβ accumulation.

Dimethyl Sulfoxide (DMSO), a Scavenger of Hydroxyl Radicals, Prevented H_2O_2-Induced Intracellular Accumulation of Aβ. It is possible that intracellular Aβ accumulation is associated with the process of cell death in general and is not caused specifically by the oxidative stress. To address the issue, we examined the effect of DMSO, a scavenger of ·OH (*24*), on the H_2O_2-induced increase in the levels of Aβ and on the cell viability. When the cells were treated with 200 μM H_2O_2, the levels of Aβ40 and Aβ42 in the Triton-soluble fraction (Figure 4A) and those of Aβ40 in the Triton-insoluble fraction (Figure 4B) were significantly increased, as described above. DMSO at 0.1% prevented these H_2O_2-induced increases in the levels of Aβ in each fraction, while DMSO treatment by itself had no effect on these levels (Figure 4). In contrast, DMSO at 0.1% only partially prevented the cell death induced by 200 μM H_2O_2 (Table 2), indicating that H_2O_2-induced cell death does not results primarily from generation of ·OH and may require an additional pathway associated with oxidative stress. Furthermore, other apoptotic stimuli including calcium ionophore and ceramide did not induce intracellular accumulation of Aβ (data not shown). These findings suggest that the oxidative stress by ·OH, and not the process of cell death, is the specific cause of intracellular accumulation of Aβ.

Table 2: Effect of Dimethyl Sulfoxide (DMSO) on H_2O_2-Induced Cell Death[a]

	MTT reduction[b]	
	% of control	% of DMSO
control[c]	100.00 ± 3.55	
H_2O_2 (200 μM)[d]	46.57 ± 1.84	
DMSO (0.1%)[c]	97.20 ± 3.07	100.00 ± 3.16
H_2O_2 + DMSO[e]	54.76 ± 3.29	56.33 ± 3.39

[a] SH-SY5Y cells were treated with 200 μM H_2O_2 for 24 h at 37 °C in the presence or absence of 0.1% DMSO. [b] Values are expressed as percentages of those in untreated cells or percentages of those in cells treated with DMSO. Data are shown as means ± sem (n = 4). [c] $p <$ 0.001, compared to H_2O_2. [d] $p < 0.05$, compared to H_2O_2 + DMSO.[e] $p <$ 0.001, compared to control.

Effect of H_2O_2 on the Expression of APP. Aβ is generated from APP through processing by two postulated proteases, the β- and γ-secretases (*25*), the former of which has been recently identified (*26*). The promoter region of APP (*27*, *28*) has the recognition motifs for NF-κB and AP-1, both of which appear to be activated by H_2O_2 (*29*, *30*). These point to the possibility that APP may be upregulated by oxidative stress. Thus, we first examined whether H_2O_2 modified the expression of the APP gene. Total RNA was prepared from the H_2O_2-treated cells and analyzed by Northern blotting using human APP cDNA as a probe. As shown in Figure 5A, there were no significant changes in the levels of APP mRNA following treatment with increasing concentrations of H_2O_2 (50−250 μM), a result consistent with a previous report (*31*).

To examine the effect of H_2O_2 on the level of full-length APP, H_2O_2-treated cells were lysed with 1% Triton X-100. The steady-state levels of full-length APP were quantitated by Western blotting using the antiserum C4 against the cytoplasmic domain of APP (*17*). C4 labeled a major band migrating at ~120 kDa and a faint band at ~110 kDa (Figure 5B). As these two bands responded in parallel to H_2O_2, we quantitated the band at 120 kDa. When the cells were treated with 50 μM H_2O_2, the levels of APP increased slightly (but not significant) (Figure 5B). Higher concentrations of H_2O_2 (≥200 μM) caused a ~35% decrease in the levels of APP (Figure 5B). In addition, the ratio of secreted (truncated) APP to intracellular full-length APP was barely affected by the H_2O_2 treatment (data not shown), suggesting that H_2O_2 did not interfere with the secretory mechanism of APP. Taken together, the present data suggest that H_2O_2 enhanced the processing of APP into Aβ in SH-SY5Y cells without affecting the expression level of APP.

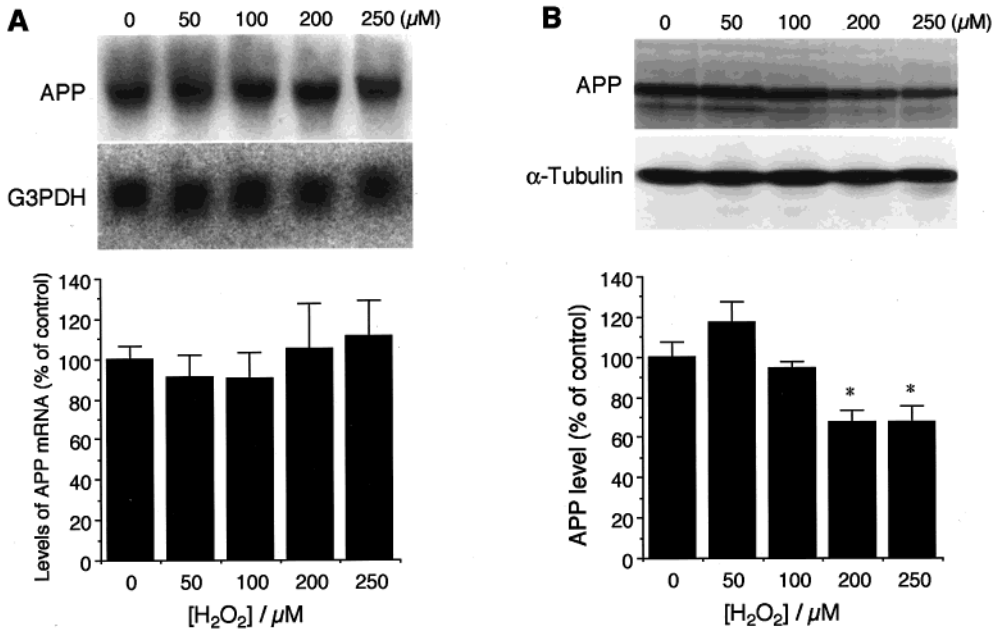

FIGURE 5: Effect of H_2O_2 on the levels of APP mRNA and full-length APP. (A) SH-SY5Y cells were treated with H_2O_2 for 24 h at 37 °C. Total RNA was prepared from the cells and separated on 1% formaldehyde-agarose gel. Blots were probed for APP or G3PDH mRNA. The signal for APP mRNA was detected at approximately 3.5 kb. (Lower panel) The levels of APP mRNA were quantitated by normalizing the values to those of G3PDH mRNA. Values are expressed as percentages (means ± sem, $n = 3$) of those in untreated cells. (B) The cells were treated with H_2O_2 for 24 h at 37 °C. The lysate solubilized with 1% Triton X-100 was analyzed for APP and α-tubulin by Western blotting with antiserum C4 against the cytoplasmic domain of APP and anti-α-tubulin monoclonal antibody. Representative blots for APP and α-tubulin are shown. The band at ~130 kDa, possibly representing mature APP, was detected after prolonged exposure (data not shown). (Lower panel) The levels of APP were quantitated by normalizing the values to those of α-tubulin. Values are expressed as percentages (means ± sem, $n = 4$) of the level in the absence of H_2O_2. *$p < 0.05$.

Effects of H_2O_2 on the Levels of the COOH-Terminal Fragments of APP. The cleavage of APP by α- or β-secretase produces two kinds of COOH-terminal fragments (stubs), C83 and C99, respectively (*25*). C99 is further processed by γ-secretase, generating Aβ (*25*), and is thus considered to be an immediate precursor of Aβ. On the other hand, C83 is a precursor of p3, Aβ17−40, and Aβ17−42. To learn more about the mechanism of intracellular accumulation of Aβ, we examined the effect of H_2O_2 on the levels of these two COOH-terminal APP fragments. To this end, both COOH-terminal fragments in the Triton-soluble and -insoluble fractions were immunoprecipitated with C4, and analyzed by Western blotting using 6E10 or C4. Immunoreactive bands were detected mainly in the Triton-soluble fraction (Figure 6) and barely in the Triton-insoluble fraction (data not shown). The antibody 6E10 labeled a major band with an apparent molecular mass of ~12 kDa and two minor bands with larger molecular masses of ~14−15 kDa (Figure 6). These three bands were also labeled with BAN50 (data not shown). Since both 6E10 and BAN50 recognize the NH_2-terminal portion of Aβ, the 12-kDa band should represent the amyloidogenic fragment C99 (calculated as having a molecular mass of 11.3 kDa). The two additional bands with larger molecular masses are presumably NH_2-terminally extended fragments (*32*). On the other hand, C4 labeled a band with an apparent molecular mass of ~8.5 kDa (Figure 4) which was also labeled by 4G8 (data not shown). The apparent molecular mass of this fragment corresponded well to that of C83 (calculated as 9.1 kDa), indicating that this is the nonamyloidogenic fragment C83. It should be noted that C4 also labeled a faint band at ~12 kDa but only after long exposure (data not shown). Another antibody to APP 643−695 labeled the 12-kDa band more strongly than C4 did (data not shown). Thus, C4 may have a weaker affinity for denatured C99 on the blot, although C4 can bind native C83 and C99 similarly, and immunoprecipitate both (see below).

Both C99 and C83 fragments were immunoprecipitated from the Triton-soluble fraction of the cells treated with 200 μM H_2O_2 for 24 h. As shown in Figure 6, H_2O_2 caused a significant (~28%) decrease in the level of C99, as compared with that in the untreated control. Comparable decreases in levels of the NH_2-terminally extended fragments were not reproducibly observed (data not shown). In contrast to C99, the levels of C83 remained the same despite H_2O_2 treatment (Figure 6). Taken together, these results indicate that H_2O_2 treatment results in lower levels of amyloidogenic C99, and strongly suggests that the treatment enhances the amyloidogenic processing of APP.

Oxidative Stress Enhanced the Amyloidogenic Processing. To confirm the above assumption, we performed a pulse-chase experiment. SH-SY5Y cells were pulse-labeled with [^{35}S]methionine, which was then chased for various lengths of time. C83 and C99 were immunoprecipitated from the Triton-soluble fraction with C4, and the levels of both fragments were quantitated. C83 and C99 were reproducibly detected as ~8.5 and ~12 kDa bands, an observation consistent with the result obtained by Western blotting (Figure 7A). On the basis of the two independent experiments, the decay rates for these two fragments were compared between control and H_2O_2-treated cells. As shown in Figure 7B, the half-life of C83 was not altered by H_2O_2 treatment: 67 min in control and 62 min in H_2O_2-treated cells. In contrast, the half-life of C99 in H_2O_2-treated cells was shorter than that in control cells: 220 min in control

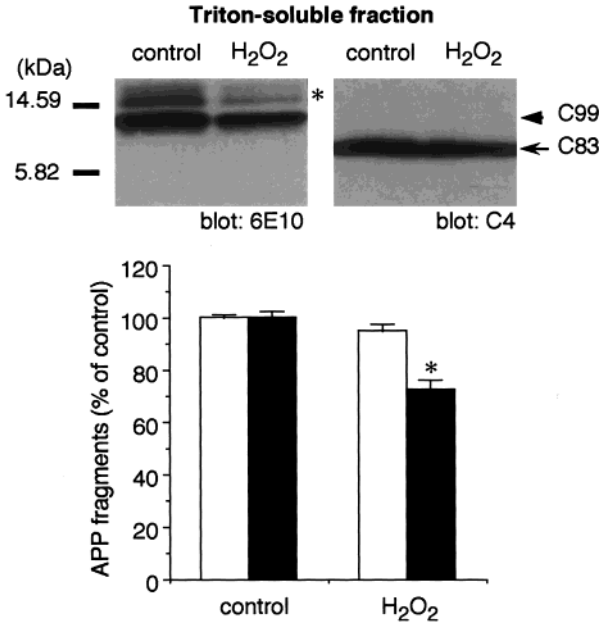

FIGURE 6: Effect of H_2O_2 on the levels of the C99 and C83 fragments. SH-SY5Y cells were treated with 200 μM H_2O_2 for 24 h at 37 °C, and Triton-soluble fractions were prepared from the cell lysate as described in the legend to Figure 2. The COOH-terminal APP fragments were immunoprecipitated with C4 and analyzed by Western blotting with 6E10 or C4. (Upper panel) Arrowheads and arrows indicate the positions of C99 and C83, respectively. The asterisk indicates possible NH_2-terminally extended fragments. The numbers on the left indicate the apparent molecular masses. Although not discernible from the figure, C99 at ~12 kDa was detected with C4 only after long exposure (data not shown). (Lower panel) The levels of C83 (open column) and C99 (closed column) in the Triton-soluble fraction were quantitated. Values are expressed as percentages (means ± sem; $n = 6$) of those in untreated control cells. *$p < 0.001$.

and 100 min in H_2O_2-treated cells (Figure 7B). These results strongly suggest that H_2O_2 treatment preferentially enhances the processing of C99 and in turn increases the production of Aβ.

DISCUSSION

Here we have shown that (i) the levels of intracellular Aβ, in particular of Aβ40, increased by H_2O_2 treatment in both neuroblastoma SH-SY5Y and CHO-APP751 cells, and (ii) the secretion of Aβ was enhanced by H_2O_2 treatment in CHO-APP751 cells. Most cells, including SH-SY5Y cells, secrete Aβ at detectable levels into the culture medium, and secreted Aβ may be taken up again into the cell, possibly through receptor-mediated endocytosis, and degraded intracellularly (*33*). Thus, the kinetics of Aβ in the intracellular compartments would also require consideration of the incorporation of Aβ secreted from the cell, thereby making the analysis most complicated. However, this possibility can be excluded primarily because our SH-SY5Y cells did not secrete Aβ at levels detectable by ELISA or immunoprecipitation (data not shown). The finding in CHO-APP751 cells strongly suggests that intracellular buildup of Aβ is not caused as a result of inhibition of Aβ secretion. In addition, secreted Aβ is generated through the endocytic pathway which appears to be relatively independent of the intracellular Aβ (*34*, *35*), although the latter is also known to be eventually secreted from the cell.

How then is intracellular Aβ accumulation induced by oxidative stress? From the above results, we hypothesize that oxidative stress causes an increased generation of Aβ through preferentially enhancing the processing of C99, the amyloidogenic fragment of APP (see below). First, the levels of APP were reduced with high concentrations (200 μM) of H_2O_2 without an associated decrease in the levels of APP mRNA. Second, a decrease in the steady-state levels of C99, the direct precursor of Aβ (*25*), was observed to occur concomitantly with an increase in the levels of Aβ. These results suggest that the processing of APP in the amyloidogenic β-cleavage pathway but not in the α-cleavage pathway is enhanced under oxidative stress. This possibility has been further strengthened by the observation that H_2O_2 treatment decreased the half-life of C99, but not that of C83, although we cannot exclude the additional possibility that oxidative stress affects specific intracellular degradative machinery and decrease the intracellular clearance of Aβ.

There are several recent reports claiming that caspases play significant roles in the amyloidogenic processing of APP (*36*, *37*). APP has several potential cleavage sites for caspase-6 or caspase-8 in the neighborhood of the NH_2-terminus of Aβ (Val-Lys-Met-Asp594, according to the numbering of APP695) and in the cytoplasmic domain (Val-Glu-Val-Asp664). Indeed, caspase-6 can cleave APP in vitro just after Asp594 and Asp664 (*37*) to generate two COOH-terminal fragments having molecular masses of 6.5 and 3.5 kDa that increase in serum-deprived neurons (*37*). These cleavages are postulated to finally generate the Aβ starting at the second position. Since Asp594 is located in the luminal portion of APP, this portion may be cleaved by β-secretase instead of caspase-6 in vivo (*38*). Either way, one may argue that in our experiments caspase-6 is activated and that it is involved in the increased production of Aβ. To examine this possibility, the cell lysate was immunoprecipitated with 4G8 and C4, and the precipitates were subjected to Western blotting with 4G8 or C4. We were, however, unable to detect these distinct fragments in the lysate (data not shown). This is consistent with the result that a buildup of intracellular Aβ occurs at low concentrations of H_2O_2 that does not affect the cell viability. Taken together, our results suggest that the accumulation of intracellular Aβ by oxidative stress is mediated mainly through enhancing the conventional β-secretase cleavage pathway.

We previously reported the presence of Aβ in the Triton-insoluble membrane compartment of SH-SY5Y cells and suggested that a distinct membrane domain, often called detergent-insoluble, glycolipid-enriched domains (DIGs) (*39*), is involved in the generation and/or trafficking of a significant fraction of intracellular Aβ40 and Aβ42 (*17*). The levels of Aβ40 in the Triton-soluble fraction were increased even with low concentrations of H_2O_2, and in a dose-dependent manner, while higher concentrations were required for an increase in the levels of Aβ40 in the Triton-insoluble fraction. C99 is abundant in the Triton-soluble fraction, suggesting that processing of C99 mainly occurs in this Triton-soluble compartment. This is compatible with previous reports of only a very small amount (or none) of APP and its COOH terminal portions being associated with DIGs (*16*, *17*, *40*). Thus, it is possible that a significant fraction of Aβ generated in the Triton-soluble compartment is incorporated into DIGs presumably in the Golgi apparatus (*39*) to be transported to

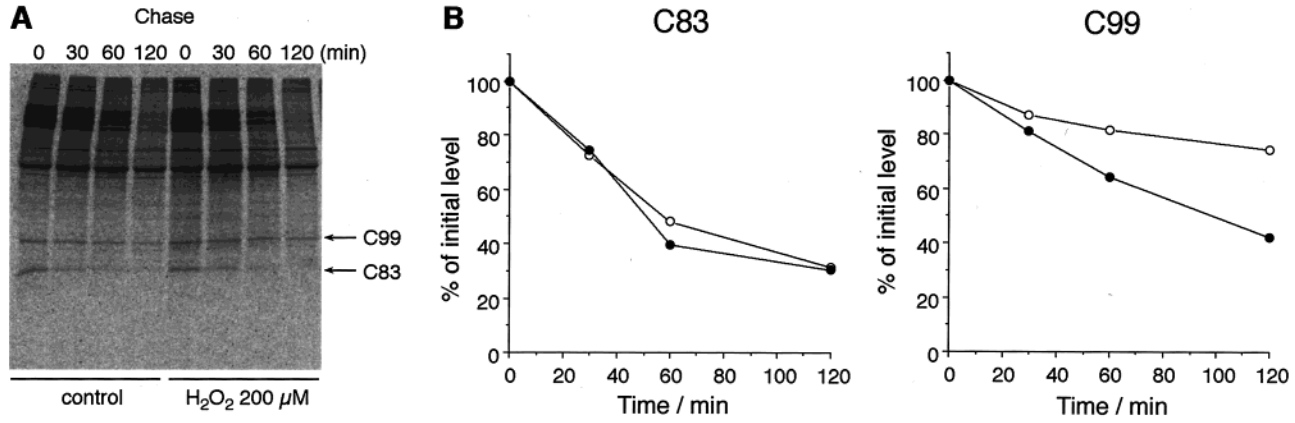

FIGURE 7: H_2O_2 enhanced the turnover rate of C99. (A) SH-SY5Y cells were treated with 200 μM H_2O_2 for 24 h at 37 °C, and labeled with [^{35}S]methionine. The cells were then chased for 30, 60, and 120 min, and homogenized in 1% Triton X-100. The COOH-terminal fragments were immunoprecipitated with C4 and subjected to SDS−PAGE. Radioactive bands were visualized (see Experimental Procedures). (B) The bands corresponding to C99 and C83 were quantitated (see Experimental Procedures). The levels of each of the fragments in untreated (open circle) and H_2O_2-treated cells (filled circle) are expressed as percentages of the levels determined immediately after the labeling. Data represent the means from two independent experiments. The decay curves could be statistically simulated by exponential regression curves ($p < 0.001$ by regression analysis), and the two decay curves of C99 were significantly different in the slopes ($p < 0.001$).

the cell surface. This incorporation into these distinct domains may take the advantage of a high affinity of Aβ for cholesterol (*41*) and ganglioside (*42*) which are present in abundance in DIGs (*43*).

Oxidative stress induces accumulation of Aβ40 to a greater extent than that of Aβ42 in both SH-SY5Y and CHO-APP751 cells. This may indicate that oxidative stress is not involved in the very initial event of β-amyloidogenesis where Aβ42 plays the major role. In this context, it is particularly important to note that, whereas an accumulation of Aβ42 is most commonly observed among aged brains in the general population, a markedly increased accumulation of Aβ40 is tightly associated with (sporadic) AD (*44*, *45*). An increased secreted pool for Aβ40 in the cell may eventually contribute to the formation Aβ40-positive plaques, and its increased intracellular pool may result in increased interaction with some cellular proteins, which may in the long term alters the energy metabolism in the cell (see below). Thus, it is possible that oxidative stress is involved in the progression of the disease process rather than in its initial stage. This assumption is consistent with repeated observations that greater extents of oxidative stress are found in AD brains (*6*, *7*, *46*).

Currently, we do not know whether a build-up of intracellular Aβ, even if small, is so harmful to the cell as to lead to degeneration or death in the long term. This could be the case, because intracellular Aβ may interact with ER-associated (ERAB)/hydroxyacy-CoA dehydrogenase (HADH) II, which was recently identified as an intracellular Aβ-binding ER protein and is involved in the metabolism of a broad range of substrates such as linear alcohols, 3-hydroxyacyl-CoA derivatives of fatty acids, and steroids (*47*). The Aβ-mediated cytotoxicity appears to be mediated by ERAB/HADH and is reported to be prevented by blocking ERAB/HADH and enhanced by its overexpression. In contrast, it may also be possible that Aβ, once built up intracellularly in the affected neurons, binds to ERAB/HADH located mainly in the ER and modulates its multiactivities and/or alters its intracellular localization (*47*), affecting the reaction with as-yet unidentified substrates and gradually leading to suppression of energy metabolism. As yet another possibility, an increase in the level of intracellular Aβ40 may be associated with an increase in the level of intracellular heparan sulfate as is postulated for those Aβ40-positive neurons in mucopolysaccharidosis (*48*), because their mutual interaction provides resistance to degradative enzymes (*49*). Thus, increased levels of heparan sulfate may help hyperphosphorylated tau in assembling into paired helical filaments (*50*), the other hallmark of AD. In addition, although only small, an increase in Triton-soluble Aβ42 may have an important significance for the neurotoxicity.

Intracellular accumulation of Aβ has been definitively shown by immunocytochemistry in vascular smooth muscle cells (*51*), hippocampal neurons in mucopolysaccharidosis (*48*), and leptomeningeal cells in AD brains (*52*). Although immunocytochemistry cannot usually detect intracellular Aβ accumulation in the neurons of AD brains, it is possible that in most neurons, Aβ40 accumulates to significant levels, thereby enhancing the progression of AD. This may be reinforced by the finding that an unusually high level of Aβ is associated with the DIGs from AD brains.[2] Future studies should address as to which Aβ compartments and which Aβ species can be harmful to the cell.

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