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Cytoprotective role of mitochondrial amyloid β peptide-binding alcohol dehydrogenase against a cytotoxic aldehyde

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Abstract

Recent reports on amyloid β peptide (Aβ) binding-alcohol dehydrogenase (ABAD) have revealed the link of Aβ with oxidative stress derived from mitochondria in the pathogenesis of Alzheimer's disease (AD). As a novel function of ABAD, we speculate that ABAD may detoxify aldehydes, such as 4-hydroxy-2-nonenal (4-HNE). To verify this speculation, we transfected cDNA encoding ABAD into cultured cells, where ABAD was localized to mitochondria. ABAD-transfectants decreased the levels of externally added 4-HNE in cultured medium as detected by TLC and became resistant against external 4-HNE. Moreover, ABAD suppressed the cytotoxic effects of cellular 4-HNE, which were produced through inducing excess reactive oxygen species (ROS) by treatment with an inhibitor of mitochondrial respiration, antimycin A. Catabolism of 4-HNE by ABAD was inhibited by Aβ, resulting in the abolishment of the cytoprotective function by ABAD against ROS. These results propose a novel role of ABAD in neural cell death in AD: ABAD detoxifies aldehydes, such as 4-HNE, derived from lipid peroxides in healthy brains, and is inhibited by Aβ in the development of AD.

Keywords: alcohol dehydrogenase; Alzheimer's disease; Amyloid β peptide; β-oxidation; oxidative stress; 4-hydroxy-2-nonenal; mitochondria

1. Introduction

Accumulation of amyloid β peptide (Aβ) has been widely accepted as a central event for the development of Alzheimer's disease (AD). On the other hand, many reports support the contribution of the decrease in energy production and the increase in oxidative stress, both of which are due to mitochondrial dysfunction [23, 25]; however, the relationship between mitochondrial dysfunction and Aβ has remained unclear for a long time. Recently, it has been revealed that some Aβ localizes to mitochondria and inhibits the activity of cytochrome *c* oxidase, a terminal enzyme of the mitochondrial electron transport chain [16, 18]. In particular, reports on the binding of Aβ to mitochondrial Aβ-binding alcohol dehydrogenase (ABAD) highlighted the molecular link of Aβ with the role of mitochondria. Aβ interacts with ABAD with high specificity and inhibits its enzymatic activity, leading to the generation of reactive oxygen species (ROS) [15].

4-hydroxy-2-nonenal (4-HNE) is widely used as a marker of excess oxidative stress, because it is an end-product derived from lipid peroxides (LPO) [13, 17]. 4-HNE is highly toxic by readily binding with lysine, histidine, serine, and cysteine residues [38]. The accumulation of LPO and 4-HNE has been reported in neurodegenerative disorders including AD [20, 31, 43].

We have previously proposed that ALDH2 is involved in the detoxification of 4-HNE generated by oxidative stress of mitochondria and that defects in ALDH2 activity cause neuronal death by stimulating the accumulation of 4-HNE due to oxidative stress [22, 26].

Alcohols [-CH_2OH] are reversibly converted into aldehydes [-CH=O] by alcohol dehydrogenases in the presence of NAD^+, while aldehydes are irreversibly converted into acids [-C(-OH)=O] by aldehyde dehydrogenases in the presence of NAD^+. As the first reaction is reversible, alcohol dehydrogenases would catalyze the reaction from aldehydes to alcohols in the presence of NADH [35]. Thus, we speculate that ABAD may function as a detoxifier of cytotoxic aldehydes and that Aβ may disturb the function leading to the accumulation of aldehydes that accelerate neuronal death. In this study, we tried to verify the working hypothesis. Here we show that Aβ inhibits the activity of ABAD to catabolize 4-HNE and abolishes the cytoprotective role of ABAD.

2. Materials and Methods

2.1. Plasmid construction,

Full-length human *HADH2* cDNA encoding ABAD was cloned from a human brain cDNA library (Gibco, Grand Island, NY, USA), which is composed of the cytomegalovirus (CMV) immediate early promoter, SV40 early mRNA polyadenylation signal, and a neomycin resistance cassette. Nucleotide sequence of ABAD cDNA was confirmed by direct sequencing.

2.1. Cell culture and constitutive transfection

HeLa cells were maintained in medium mixed with Dulbecco's modified Eagle's medium and Fam-12 (DMEM/F-12) containing 10% fetal bovine serum (FBS). ADAD cDNA was transfected into HeLa cells with PolyFect® Transfection Reagent (Qiagen, Valencia, CA, USA) after digestion of *Apa LI* for linearization. The cells were selected using 400 μg/mL of Geneticin® (Gibco) to obtain constitutive transfectants.

2.2. Immunostaining

Cultured cells were placed on 4-well plastic plates (SonicSeal slide; Nalge Nunc, Rochester, NY, USA) at 4 x 10^4 cells/mL (HeLa cells) or 1 x 10^5 cells/mL (SHSY-5Y cells), and continued to culture for 24 hr. When ABAD was imaged, HeLa or SHSY-5Y transfectants were first treated with a fluorescent indicator for mitochondria, MitoTrackerRed (Molecular Probes, Eurogene, OR, USA) (100 nM or 500 nM) for 10 min, followed by immunostaining with anti-ABAD antibody. When peptides conjugated with 4-HNE were imaged, cells were cultured with antimycin A for 24 h in medium containing 1% FBS instead of 10% as described previously [22], followed by immunostaining. For immunostaining, cells were rinsed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 30 min and incubated for 30 min in 0.2% Triton-X 100, and then cells were soaked for 30 min at room temperature in a blocking buffer (3% bovine serum albumin and 3% goat serum in PBS), and incubated for 1 hr at 37 °C in a blocking buffer containing monoclonal anti-ABAD antibody (ERAB; 1:250, BD Transduction Laboratories, Franklin Lakes, NJ, USA) or overnight at 4 °C with monoclonal anti-4-HNE antibody (HNEJ-2; 10 μg/mL, Japan Institute for Control of Aging, Shizuoka, Japan). After incubation and another wash with PBS, cells were incubated in blocking buffer containing BODIPY FL goat anti-mouse IgG (1:500, Molecular Probes) for 1 hr. Anti-ABAD was imaged by confocal scanning microscopy using excitation and emission filters of 488 nm and 520 nm, respectively. Cells stained with MitoTrackerRed were imaged using excitation and emission filters of 543 nm and 565 nm, respectively. Average pixel intensity stained with anti-4-HNE antibody was measured in each cell and expressed in relative units of fluorescence.

2.3. Immunoblotting

HeLa cells was harvested, washed with PBS twice, disrupted with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, and 0.2% sodium dodecyl sulfate and protease inhibitor cocktail tablets (Roche, Mannheim, Germany)) and centrifuged at 10,000 x g for 10min. The protein concentration was determined by Bio-Rad bicinchoninic protein assay (Bio-Rad, Hercules, CA, USA), and then samples (10 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis, followed by electrophoretical transfer to a polyvinylidene difluoride membrane. The membrane was incubated for 1 hr in a blocking buffer (5% dry-fat skim milk and 0.1% Triton X-100 in Tris-buffered saline), and overnight at 4 °C in a blocking buffer containing monoclonal anti-ABAD antibody (ERAB; 1:1000) or anti-actin antibody (Sigma). After several washes with TBST, the membrane was incubated in a blocking buffer containing AP-conjugated sheep affinity-purified F(ab')2 fragment to mouse IgG (1:10000, ICN, CA, USA) for 1 hr. After several washes, the

membrane was incubated with AttoPhos Substrate Set (Boehringer Ingelheim, Ingelheim, Germany) with imaging by BAS-2000II (Fuji Photo Film, Kanagawa, Japan) using excitation and emission filters of 420 nm and 560 nm, respectively.

2.4. 4-HNE treatments

4-HNE (Calbiochem, San Diego, CA, USA) was dissolved in DMSO as a stock solution and, just before use, diluted with Kreb-Henseleit buffer supplemented with 11.5 mM Hepes-Na, pH 7.2. Transfectants were placed on a 9 cm dish at a density of 2 x 10^4 cells/cm^2 and the medium was exchanged with 1 mL of Kreb-Henseleit buffer containing 250 μM 4-HNE. After incubation at 37 °C for 15 or 30 min, external 4-HNE was extracted three times from the supernatant with 5-fold dichloromethane, dried, and resuspended in dichloromethane. The whole extract was spotted onto a silica thin layer chromatography (TLC) plate (Analtec, Inc. Newark, DE, USA) and developed with acetone/hexane (30:70, v/v). 4-HNE was detected after heating with methanol/sulfuric acid (1:1, v/v) and identified according to the migration with standard 4-HNE (Rf = 0.49). When Aβ-pretreatment is necessary, the Aβ peptide ($A\beta_{1-42}$ (human), Biosource, Camarillo, CA, USA) was incubated for 4 days at 37 °C to be aggregated in PBS and added to cell culture to 1 μg/mL in DMEM/F12 containing 1% FBS for 14 hr at 37 °C.

To examine cell viability, cells were placed in 24-well plates at a density of 2 x 10^4 cells/cm^2, treated with 10 μg/mL 4-HNE in DMEM/F-12 medium containing 1% FBS for 24 hr, followed by staining with 10 μM propidium iodide (PI; to detect nuclei of dead cells) and 10 μM Hoechst 33342 (for nuclei of total cells). Dead and living cells were enumerated from over 100 cells under a fluorescence microscope in a blinded fashion. When necessary, pretreatment with aggregated Aβ was performed as above.

2.5. Antimycin A or H_2O_2 treatment

Transfectants were plated on 24-well plates at a density of 2 x 10^4 cells/cm^2. Antimycin A was dissolved in DMSO to adjust to the desired concentration, and just before use, diluted 1,000-fold with DMEM/F-12 containing 1% FBS. H_2O_2-treatment was performed for 24 hr. Cell viability was examined as described above.

2.6. Resistance of transient transfectants against 4-HNE-treatment

SHSY-5Y cells were maintained in Dulbecco's modified Eagle's medium containing 15% FBS. One day before transfection, SHSY-5Y cells were plated in a 60-mm dish at density of 1 x 10^5 cells/cm^2 in 15% FBS in DMEM. Cells were co-transfected with 4 μg of pEGFP-N1 (Clontech) as an EGFP marker for transfected cells and 12 μg of cDNA constructs (ABAD or vector) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Under this condition, most transfectants expressing EGFP should express ABAD. The transient transfected cells were trypsinized and re-plated at density of 5 x 10^4 cells/cm^2. After incubated overnight, the cells were washed with Hank's at once, and treated with 4-HNE in 1% FBS in DMEM for 24 hr. EGFP-positive living cells were enumerated under a fluorescence microscope from 40 randomly selected fields (x200).

2.7. Statistical analysis

Statistical analyses were performed using StatView software (SAS Institute). Unpaired two-tailed Student *t*-test and ANOVA followed by Fisher's exact test were used for single and multiple comparisons, respectively. Experiments for quantification were performed in a blinded fashion.

3. RESULTS

3.1. ABAD localizes to mitochondria

To reveal the role of ABAD in living cells, we transfected human ABAD cDNA under a CMV promoter into HeLa cells to overexpress ABAD. ABAD is ubiquitously expressed to function in the third step of β-oxidation in most cells as will be noted in Discussion; however, HeLa cells are poor in β-oxidation [24]. Thus, HeLa cells were chosen as a first target because, even when the enzyme involved in β-oxidation is overexpressed, energy metabolism would not be disturbed.

After the expression of ABAD was tested by Western blotting (Fig. 1A), two control clones (clones V1 and V2) and two ABAD-positive clones (clones A1 and A2) were used throughout this study. We detected ABAD with its specific antibody by confocal laser scanning microscopy: the majority of ABAD localized to mitochondria (Fig. 1B), which is in good agreement with previous reports [7, 15], whereas transfectants with empty plasmid exhibited less ABAD (Fig. 1C).

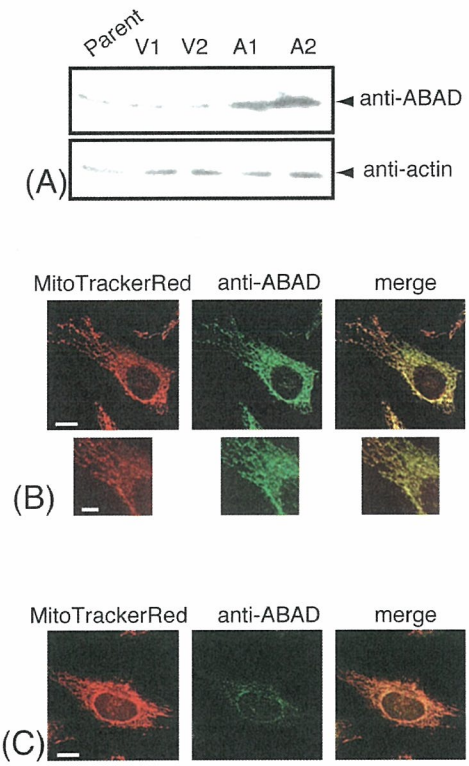


Fig. 1. ABAD localizes to mitochondria. (A) Western blot of control and ABAD-transfectants. Samples of 10 μg protein were subjected to Western blotting stained with anti-ABAD or anti-actin antibody as an internal control. Lanes V1 and V2 indicate control-transfectants with an empty vector; and lanes A1 and A2, ABAD-transfectants. (B) Representative images of an ABAD-transfectant (A1) costained with MitoTrackerRed (left panels) anti-ABAD (middle panels) and superimposed (right panels). Scale bar: 10 μm (upper panels) and in 5 μm (magnification shown in lower panels). (C) Representative images of a control-transfectant (V1) costained with MitoTrackerRed (left panels) anti-ABAD (middle panels) and superimposed (right panels). Scale bar: 10 μm

3.2. ABAD decreases externally added 4-HNE

First, we examined whether ABAD-transfectants catabolize external 4-HNE (250 μM), the amount of which can be detected by TLC. ABAD-transfectants and control cells were exposed to external 4-HNE in a limited volume of culture medium for the indicated periods, then 4-HNE was extracted from the media with

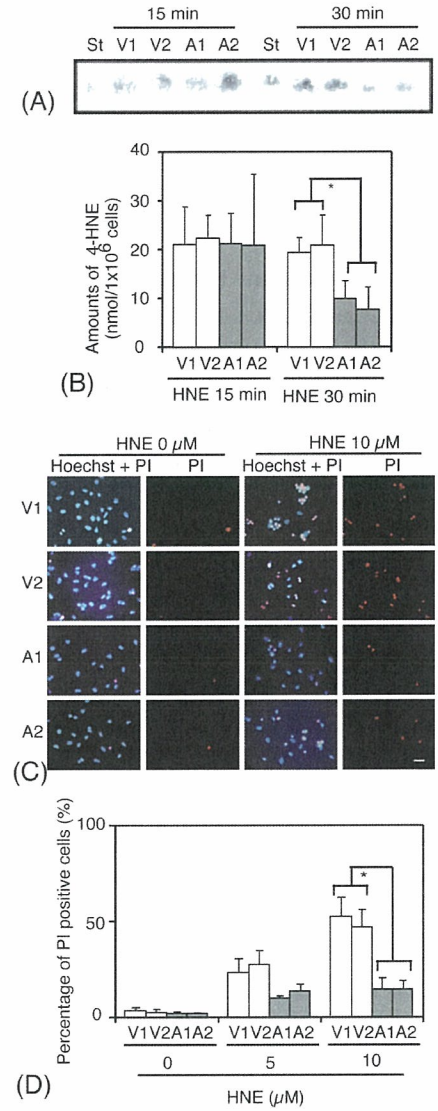


Fig. 2. ABAD decreases amounts of externally added 4-HNE. (A) Representative patterns of TLC for quantifying 4-HNE. Each transfectant was treated externally with 4-HNE (250 μM) for the indicated periods, extracted from the supernatant medium, spotted onto TLC, and visualized as described in **Materials and Methods**. Lanes indicate control- (V1 and V2) and ABAD-transfectant (A1 and A2). St indicates a spot of standard 4-HNE (12.5 nmol). (B) Intensities of spots with Rf = 0.49 quantified with NIH image to calculate the amounts of 4-HNE. Lanes indicate clones shown in (A). Data are shown as the mean ± SD from four independent experiments. *$p<0.05$ in Student's t-test. (C) Representative fluorescent pictures of nuclei of each transfectant stained with Hoechst33342 (blue: dead and living cells) and PI (pink: dead cells) after treatment with or without 10 μM 4-HNE for 24 hr as described in **Materials and Methods**. Scale bar: 50 μm. (D) Percentage of dead cells of each transfectant after treatment with the indicated concentration of 4-HNE. Total and dead cells were enumerated under a fluorescent microscope. Lanes indicate vector control (V1 and V2) and ABAD transfectant (A1 and A2). Data are the mean ± SD of 4 independent experiments and *$p<0.05$ in Student's t-test.

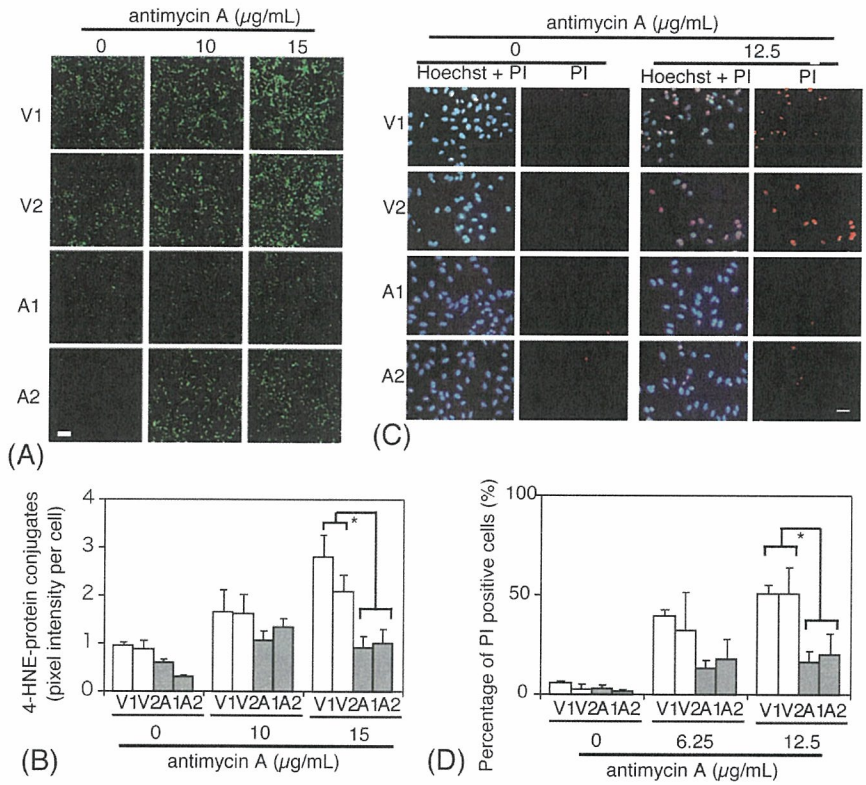


Fig. 3. ABAD catabolizes 4-HNE generated through ROS. (A) Representative images of immunostaining to 4-HNE conjugated with protein after treatment with antimycin A. Control- (V1 and V2) and ABAD- (A1 and A2) transfectants were treated with the indicated concentrations of antimycin A for 24 h and imaged by confocal laser scanning fluorescent microscopy. After fixation, cells were stained with anti4-HNE antibody. Scale bar: 200 μm. (B) 4-HNE-protein conjugates quantified from pixel intensity in cells stained with anti-4-HNE antibody. Pixel intensity was measured with NIH image. Lanes indicate V1 and V2 (control-transfectant) and A1 and A2 (ABAD-transfectant). Data are the mean ± SD and * $p<0.05$ in Student's t-test. (C) Representative fluorescent pictures of nuclei of each transfectant stained with Hoechst33342 (blue: dead and living cells) and PI (pink: dead cells) after treatment for 24 hr with or without 12.5 μg/mL antimycin A for 24 hr as described in **Materials and Methods**. Scale bar: 50 μm. (D) Percentage of dead cells of each transfectant after treatment with the indicated concentrations of 4-HNE. Total and dead cells were enumerated under a fluorescent microscope. Lanes indicate V1 and V2 (control-transfectant) and A1 and A2 (ABAD-transfectant). Data are the mean ± SD of 4 independent experiments and *$p<0.05$ in Student's *t*-test.

dichloromethane and subjected to TLC, followed by visualization by heating. Thirty minutes after exposure, external levels of 4-HNE in the media were significantly decreased only in ABAD-transfectants (Fig. 2A, B).

Next, the cytoprotective effects of ABAD were examined against 4-HNE. One day after treatment with 10 μM 4-HNE, ABAD- and control-transfectants were stained with 10 μM PI (pink for dead cells) and/or 10 μM Hoechst 33342 (blue for dead and living cells) to distinguish dead and living cells. Considerable dead cells were found in control-transfectants in a dose-dependent manner, whereas fewer dead cells were seen in ABAD-transfectants (Fig. 2C, D). Thus, these experiments suggest that ABAD catabolizes 4-HNE to protect cells against the cytotoxicity of 4-HNE.

3.3. ABAD catabolizes 4-HNE induced through ROS

Since 4-HNE rapidly modifies proteins, the possibility may not be ruled out that the decrease in 4-HNE in the media may be due to only the acceleration of incorporation of 4-HNE. As 4-HNE is produced from lipid peroxides in a non-enzymatic manner, we were forced to generate superoxide radicals by treatment with a mitochondrial respiratory inhibitor, antimycin A [33], and then conjugated proteins with 4-HNE were detected by immunostaining using a specific antibody. The amount of conjugated proteins with 4-HNE increased after treatment with antimycin A in controls (Fig. 3A, B), while it did not increase in ABAD-transfectants (Fig. 3A, B); therefore, it is concluded that ABAD catabolizes 4-HNE.

The protective ability against cell death was examined

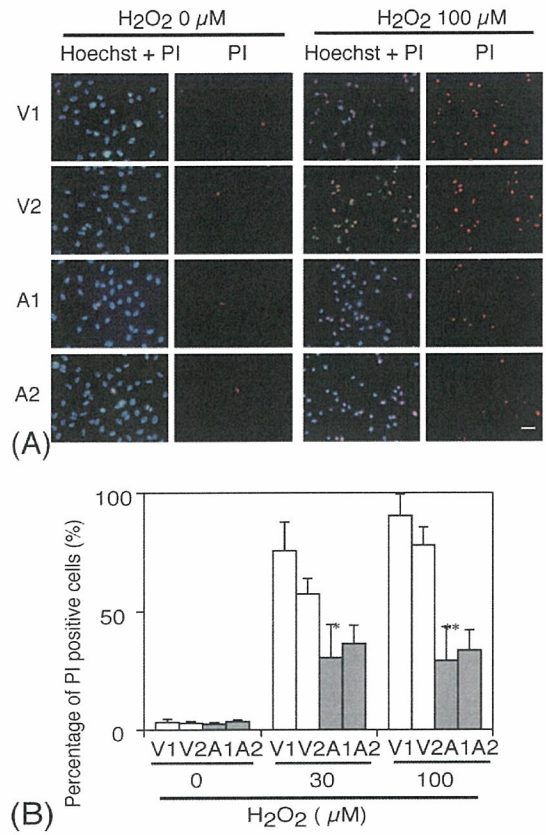


Fig. 4. ABAD-transfectants are resistant to oxidative stress induced by H_2O_2. (A) Representative fluorescent pictures of nuclei of each transfectant stained with Hoechst33342 (blue: dead and living cells) and PI (pink: dead cells) after treatment for 24 hr with or without 100 μM H_2O_2 for 24 hr as described in **Materials and Methods**. Scale bar: 50 μm. (B) Percentage of dead cells of each transfectant after treatment with the indicated concentrations of H_2O_2. Total and dead cells were enumerated under a fluorescent microscope. Lanes indicate V1 and V2 (control-transfectant) and A1 and A2 (ABAD-transfectant). Data are the mean ± SD of 4 independent experiments and *p<0.05 in Student's *t*-test.

by treatment with antimycin A. Considerable dead cells in controls were seen after antimycin A treatment, while ABAD-transfectants were resistant to the treatment (Fig. 3C, D).

As an alternative oxidative stress, we examined effects by hydrogen peroxide (H_2O_2). Similarly to 4-HNE- and antimycin A-treatments, ABAD-transfectants were more cytoprotective to the oxidative stress than controls (Fig. 4A, B).

3.4. Cytoprotective role of ABAD is suppressed by Aβ

When cell cultures are exposed to $A\beta_{1-42}$, $A\beta_{1-42}$ binds to ABAD [40]. Thus, we examined whether Aβ actually inhibits ABAD activity in the detoxification of 4-HNE. When cells were treated with $A\beta_{1-42}$, it inhibited the decrease in external 4-HNE only in ABAD-transfectants (Fig. 5A, B). Moreover, $A\beta_{1-42}$ inhibited the cytoprotective role by ABAD against the exposure to ROS induced through treatment with antimycin A (Fig. 5C). It was reported that co-overexpression of ABAD and mutant APP induced cytotoxicity [40]. In contrast to this report [40], no cytotoxic effect was observed even in the presence of Aβ without treatment with antimycin A (Fig. 5C). Only when cells were co-treated with Aβ and antimycin A, the difference between ABAD- and control-transfectants was evident. This result suggests that ABAD protects cells by detoxifying 4-HNE.

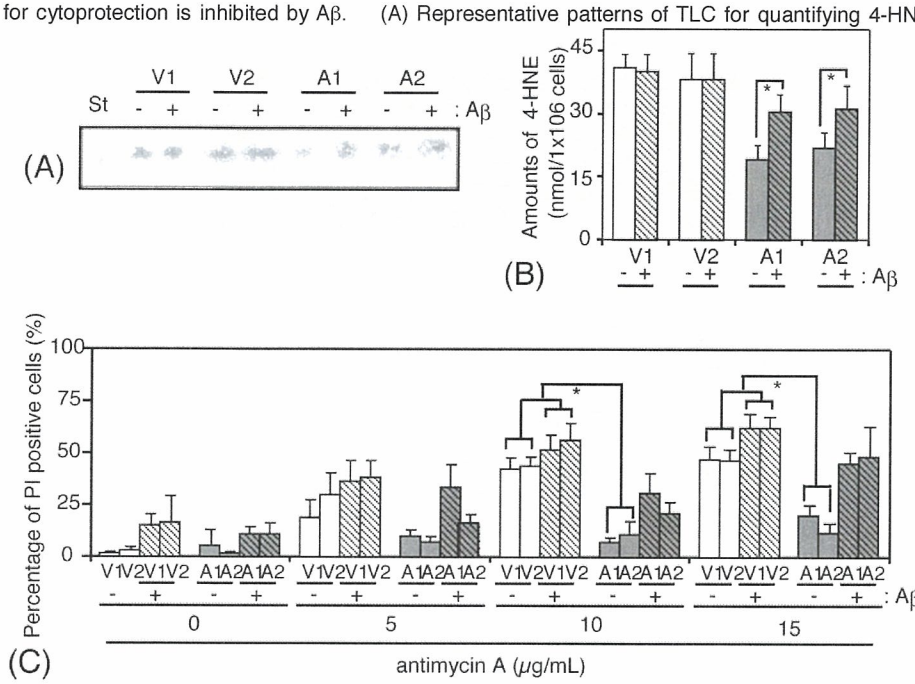
3.5. ABAD is cytoprotective against 4-HNE in neuroblastoma cells

Finally, we examined the cytoprotective role of ABAD against 4-HNE in neuroblastomas. SHSY-5Y cells were transiently transfected with ABAD cDNA and imaged with with Mitotraker red and anti-ABAD antibody (Fig. 6A), confirming that ABAD localizes to mitochondria. Then , SHSY-5Y cells were co-transfected with the ABAD and EGFP genes and treated with 4-HNE as described in **Materials and Methods**. EGFP-positive cells were enumerated under fluorescence microscope after 4-HNE-treatment for 24 hr. The 4-HNE-treatment decreased the EGFP-positive cells due to cell death (Fig. 6B, C). Apparently, vector/EGFP co-transfectants were less than ABAD/EGFP (Fig. 6B, C), indicating ABAD-transfectants were more resistant against the 4-HNE-treatment. These results suggested that ABAD catabolizes 4-HNE and protected from cell death in neuroblastoma cells.

4. Discussion

Oxidative stress is widely accepted as one of the causes of neurodegenerative disorders including AD. Oxidative stress arises from the strong cellular oxidizing potential of excess ROS. The majority of superoxide anion radicals is generated in mitochondria by electron leakage from the electron transport chain [25]. Antimycin A inhibited smooth electron transport at complex III, and induced the production of superoxides. Superoxides may be converted into hydrogen peroxide, which is a source of the most reactive radical, hydroxyl radical [33]. ROS modifies unsaturated fatty acids to form

Fig. 5. ABAD activity for cytoprotection is inhibited by Aβ. (A) Representative patterns of TLC for quantifying 4-HNE. Transfectants



were pretreated with 1 μg/mL Aβ for 14 hr and exposed to external 4-HNE (250 μM) for 30 min. External 4-HNE was extracted from the supernatant medium, spotted onto TLC and visualized as described in Materials and Methods. Lanes indicate control (V1 and V2) and ABAD transfectants (A1 and A2). Aβ + and – indicate with and without preincubation with aggregated Aβ. St indicates a spot of standard 4-HNE (12.5 nmol). (B) Intensities of spots with Rf = 0.4 quantified with NIH image to calculate the amounts of 4-HNE remaining in the supernatant. Lanes are shown as in (A). Data are shown as the mean ± SD of four independent experiments. *$p<0.05$ in Student's t-test. (C) Percentage of dead cells of each transfectant after pretreatment with Aβ for 14 hr, followed by treatment with antimycin A for 24 hr. After treatment, cells ware stained with PI (red) and/or Hoechst33342 (blue). Total (blue) and dead cells (pink) were enumerated under a fluorescent microscope. Lanes are shown as in (A). Data are the mean ± SD of 4 independent experiments and *$p<0.05$ in Student's t-test.

peroxides, from which aldehydes such as marondialdehyde (MDA), and highly toxic 4-HNE are non-enzymatically produced. In particular, 4-HNE denatures proteins by modifying lysine, histidine, serine, and cysteine residues [38]. It has also been shown *in vitro* to promote neuronal death [11]. Recently, marked increases in 4-HNE were reported in the hippocampus and superior and middle temporal gyrus of patients with mild cognitive impairment (MCI) and those with early AD compared with healthy individuals [39]. 4-HNE not only induces neuronal death but also causes synapse dysfunction due to mechanisms such as reducing Na^+,K^+-ATPase activity [27] and markedly inhibits microtubule formation and neurite outgrowth [21]. Furthermore, there have been a number of reports on the relationship between neurofibrillary tangle (NFT), which is a pathological feature characteristic of AD, and oxidative stress [44]. Concerning 4-HNE in particular, it has been reported to induce structural changes in phosphorylated tau by modifying it and to make tau a structure in NFT [12, 36], so that 4-HNE is considered to play an important role in NFT formation. In a transgenic mouse model of Aβ deposition, LPO accumulation was reported to precede Aβ accumulation [30].

Many enzymes may catabolize 4-HNE for detoxification as follows: aldo-keto oxidoreductases; ALDH; aldosereductase; aldehyde reductase; and alcohol dehydrogenase (ADH) [29]. ALDH2-deficient transfectants were vulnerable to exogenous 4-HNE and accumulated endogenous 4-HNE by treatment with antimycin A [22, 25, 26], which supports our epidemiologic case-control study that mitochondrial ALDH2 deficiency is a risk for late-onset AD [8].

(A) Aβ has recently been shown to exist inside mitochondria, and inhibits the activity of cytochrome *c* oxidase [1, 4, 34]. Moreover, the link of mitochondria with Aβ was revealed by attractive findings that Aβ binds to ABAD to inhibit its activity, resulting in the excess generation of ROS. A mutant APP gene and the ABAD gene were introduced into transgenic mice to enhance Aβ

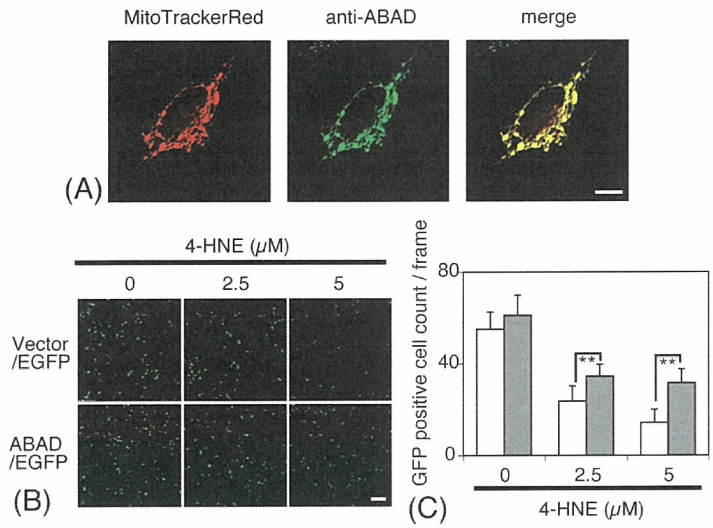


Fig. 6. Neuroblastomas expressing ABAD were resistant against 4-HNE.
(A) SHSY-5Y cells were transiently transfected with ABAD cDNA. Representative images of a transfectant co-stained with MitoTrackerRed (left panel) anti-ABAD (middle panel) and superimposed (right panel) are shown. Scale bar: 10 μm (B) SHSY-5Y cells were transiently co-transfectioned with EGFP/vector or EGFP/ABAD, treated with the indicated concentration of 4-HNE for 24 hr and observed with a fluorescent microscope. Scale bar: 200 μm. (C) Living cells expressing EGFP were enumerated after 24h treatment with 4-HNE under a fluorescent microscope. Data are the mean ± SD of 4 independent experiments. **p<0.01 in Student's t-test.

production. In transgenic mice, an increase in oxidative stress in neurons was accompanied with memory loss [15].

ABAD was initially reported as endoplasmic reticulum-associated amyloid β-peptide (Aβ)-binding protein (ERAB) [40]. Although there was discrepancy about the location of ABAD in the early stage, this enzyme was accepted to be located in mitochondria [7]. This study confirmed that ABAD localizes to mitochondria.

In this study, we hypothesized that ABAD may have an additional function that catabolizes 4-HNE. Indeed, we verified this hypothesis at least at cultured cell level, although it remains unclear whether ABAD actually functions to detoxify cytotoxic aldehydes in the brain.

ABAD has several functions: in the third step of mitochondrial fatty acid β-oxidation, cycles comprised four sequential reactions, as short chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) [5, 10, 14]: additionally, ABAD catalyzed a wide spectrum of substrates, including steroids [6, 7, 41, 42], cholic acids [6, 42], and fatty acid. Thus, if this multifunctional enzyme would have an additional function, it might be reasonable. When we pay attention on mitochondrial fatty acid β-oxidation [32], the β-oxidation is not available in energy metabolism in the brain [2, 3,19, 28]; however, ABAD, an enzyme involved in the β-oxidation, expresses in the brain [42]. Thus, it suggests that ABAD plays an alternative role in the brain instead of energy metabolism.

ABAD can detoxify 4-HNE only in the presence of NADH as a cofactor in the healthy brain according to our model. Thus, when energy metabolism to generate NADH is declined, the detoxification system by ABAD would not be functional, leading to amplify toxic aldehydes. Since it is known that energy metabolism is poor in the brain of AD, NADH must be not abundant in AD brains. Additionally, activation of poly(ADP-ribose) polymerase-1 has been recently found in AD [9], which consumes NAD^+ to form branched polymers of ADP-ribose on target proteins. Thus, NAD^+ as well as NADH must be exhausted in the brain with AD, which leads to dysfunction of ABAD for detoxifying aldehydes.

In our model, Aβ plays a role towards the accumulation of 4-HNE by inhibiting the ABAD activity in the development of AD. Since 4-HNE stimulates the Aβ production [37], Aβ would in turn enhance to increase by 4-HNE. This vicious cycle could increase Aβ as well as 4-HNE, both of which should contribute to the pathogenesis of AD. Further study will be required to reveal the relationship between AD and ABAD.

Acknowledgments

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総説

アルツハイマー病の危険因子である酵素活性欠損型アルデヒド脱水素酵素2遺伝子

——その分子メカニズムとモデル動物の開発——

大澤郁朗，太田成男

はじめに

アルツハイマー病（AD）をはじめとする加齢に伴う神経変性疾患の発症に対してもっとも普遍的な危険因子は老化である．しかし，老化がなぜ危険因子となるのかについての明確な答えはない．細胞を構成する蛋白質，核酸，脂質などの物質が年数を経るにつれ変性し，それが細胞及び個体の変性，すなわち老化を引き起こすとされている．その変性を引き起こす主体となるものが酸化ストレスであることが明らかとなってきた．生命体は，その維持と活動に必要な代謝エネルギーを効率良く生み出すために27億年の昔から酸素を利用するようになった．真核細胞においては，主にミトコンドリアで85～90％の酸素がエネルギー代謝に用いられる．しかし，その過程では常に電子の漏れが生じ，その電子によって酸素が還元されて活性酸素種（ROS）となり，細胞に酸化ストレスを与える．その為，ミトコンドリアの内外でこの酸化ストレスを除去する機構が存在し，常に細胞を防御している．この防御機構が疲弊すると老化は促進される．従って，ADなどの神経変性疾患が発症する危険性が増大することが予想される．

本稿では，アルコール代謝に関わっているとされてきたミトコンドリアのアルデヒド脱水素酵素2（ALDH2）が，酸化ストレスによる脂質の過酸化で派生する毒性の高いアルデヒド類を除去する酵素であることを概説し，ALDH2酵素活性欠損型遺伝子の保持がADの危険因子である原因を分子レベルで論じる．さらにALDH2活性を抑制したトランスジェニック・マウスでは加齢に伴う神経変性と空間的学習能力の低下が認められたので，その一部について紹介する．

The deficiency in a mitochondrial aldehyde dehydrogenase as a risk factor of Alzheimer's disease: Its molecular mechanism and a model animal
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1. 遺伝子多型によって生じるドミナント・ネガティブ型ALDH2

アルデヒド脱水素酵素はヒトにおいて少なくとも16個の異なる遺伝子からなる大きなファミリーを形成しており，その発現分布と基質特異性から，それぞれが多様なアルコール，アルデヒド代謝系に組み込まれている（Vasiliou & Pappa, 2000）．この中で，*ALDH2* 遺伝子は染色体12q24.2上にあり，ミトコンドリアのマトリックスに局在するホモ4量体からなる酵素をコードする．ALDH2には活性型のALDH2*1と不活性型のALDH2*2が存在し，両者の構造的な違いは1塩基置換によって487番目のグルタミン酸がリジンに置き換わった点である（Yoshida, et al, 1984）．ALDH2*1からなるホモ4量体のうち一つでもALDH2*2に置き換わると，構造変化によって補酵素であるNAD^+との結合能が低下して酵素活性が失われる（Larson, et al, 2005）．すなわち，ALDH2*2はドミナント・ネガティブに働き，仮にALDH2*1とALDH2*2が同じ割合で存在すれば，酵素活性は1/16となる（図1）．このALDH2は低濃度のアセトアルデヒドを基質とすることができ，飲酒時においてエタノールがアルコール脱水素酵素（ADH）によって酸化されることで生じるアセトアルデヒドをALDH2がさらに酸化して酢酸とし，エネルギー源に変えることができる．この為，ALDH2活性が低いと飲酒時にアセトアルデヒドが蓄積し，紅潮，悪心，頻拍といったいわゆる“お酒に弱い人”に特徴的な症状を呈する．不活性型の *ALDH2*2* アレルを保有するのは東アジア系人種に限定され，日本人の場合は約40%が *ALDH2*2* アレルを一つ以上保有し，さらに約10%が *ALDH2*2* ホモでALDH2活性をまったく持たない（Takeshita, et al, 1994）．

2. *ALDH2*2* アレルは晩期発症型アルツハイマー病の危険因子

65歳以上で発症したAD患者472名と対照群として非認知症者472名の *ALDH2* 遺伝子

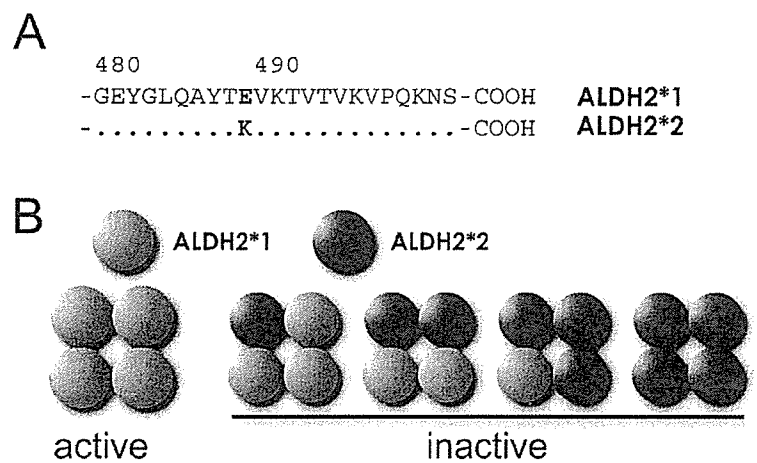

図1. *ALDH2* 遺伝子多型
A：ALDH2のC末端アミノ酸配列．*ALDH2* 遺伝子の1塩基置換により，活性型（ALDH2*1）と不活性型（ALDH2*2）になる．B：ALDH2は単一サブユニットからなる4量体を形成する．サブユニットのうち1つでも不活性型になると酵素活性は失活するため，ALDH2*2はドミナント・ネガティブに働く．

表 1.　AD 患者と対照者の *ALDH2* 遺伝子型頻度

Subjects	Number of genotype [frequency]			
	1/1	*1/2*	*2/2*	*1/2 & 2/2*
Patients (n=447)	232 [0.519]	183 [0.409]	32 [0.072]	215 [0.481]*
Controls (n=447)	280 [0.626]	138 [0.309]	29 [0.065]	167 [0.374]

*ALDH2*1* アレルと *ALDH2*2* アレルの頻度は AD 患者で 0.724 と 0.276 であったのに対し，対照者では 0.781 と 0.219 であった ($p=0.005$)．*$p=0.001$, OR=1.6 (95% C.I.=1.19-2.03)．

多型を解析した (Kamino, et al, 2000)．*ALDH2* 遺伝子多型は各国で比率が大きく異なるが，日本国内でも地域差がある．また，老年病に関わる遺伝子多型の頻度は高齢になるにつれ変化することも予想される．そこで，対照群は，男女比，年齢のみならず地域についてもこれをそろえて比較した．その結果を表 1 に示す．AD 患者では少なくとも一つ以上 *ALDH2*2* アレルを保有する者の割合が 48.1% であったのに対し，非認知症者では 37.4% に過ぎなかった．この時のオッズ比は 1.6，p 値は 0.001 で十分に有意である．男女別々に解析しても同様の結果となり，性差は認められなかった．

さらに晩期発症型 AD の危険因子として知られるアポリポ蛋白 E (ApoE) について解析したところ，*APOE ε4* アレルの AD 発症頻度に対するオッズ比は 3 であった．この APOE 遺伝子多型と ALDH2 遺伝子多型を組み合わせて比較した結果を図 2 に示す．この結果から，*APOE ε4* と *ALDH2*2* の両アレルを共に保有する場合には AD 発症頻度が相乗的に高くなることが判明した．とくに *APOE ε4* ホモで少なくとも一つ以上の *ALDH2*2* アレルを保有する者は，どちらも保有していない者に比べ，31 倍も AD 発症頻度が高い．この条件に一致する者は日本人の約 1% と推定され，計算上，ほぼ確実に AD を発症することになる．また，*APOE ε4* と *ALDH2*2* の相乗効果により発症年齢も有意に早くなっていた．

これらの結果は，病理学的に確定診断した後の患者試料を用いた筑波大の玉岡らの研究でも *ALDH2*2* アレルが AD の危険因子であること，*APOE ε4* による相乗効果があることについて再現性が確認されている (Tamaoka, et al,

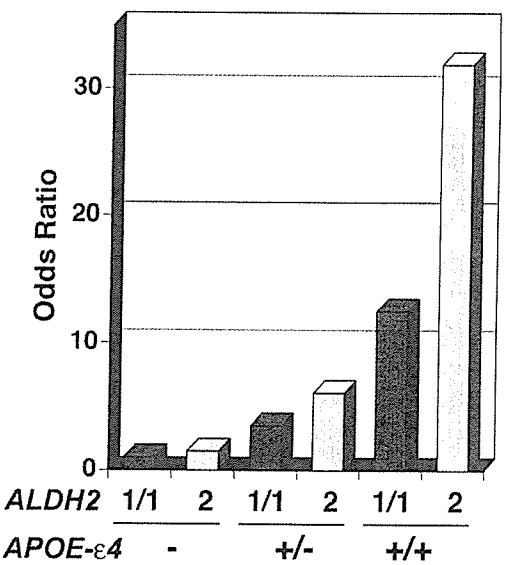

図 2.　孤発性アルツハイマー病における *ALDH2*2* と *APOE ε4* の発症危険性にたいする相乗効果
ALDH2 の (1/1) は *ALDH2*1* アレルをホモで保持する者を (2) は *ALDH2*2* アレルをヘテロまたはホモで保持する者を表す．*APOE ε4* の (-) は *ε4* アレルを保持しない者を，(+/-) はヘテロで，(+/+) ホモで保持する者をそれぞれ表す．*ALDH2*2* アレルを持ち，*APOE ε4* アレルがホモの者では，どちらも保持しない者に対して，AD 発症のオッズ比が 31 倍になる．

2003 日本痴呆学会)．一方，韓国でもADの危険因子として*ALDH2*遺伝子多型が着目されて解析が行われたが，認知能力とALDH活性の間には相関がないと報告されている(Kim, et al, 2004)．しかし，この報告ではAD患者わずか60名について解析したが統計的な有意差が認められなかったとしており，十分な数を検討したとは言えない．

3. *ALDH2*2*アレルと酸化ストレスの増大

*ALDH2*遺伝子多型はアルコールに対する感受性を大きく変えることから，*ALDH2*2*アレルを保持する人はアルコール依存症やアルコール性肝炎などの過度のアルコール摂取による疾患に罹患している率は低い(Goedde, et al, 1992)．一方，*ALDH2*2*アレルは糖尿病，腫瘍，高血圧，心筋梗塞の危険因子であることも報告されている(Suzuki, et al, 1996; Yokoyama, et al, 1998; Takagi, et al, 2001; Amamoto, et al, 2002)．しかし，*ALDH2*の遺伝子多型は飲酒という生活習慣と密接に関連しているので，遺伝子多型の影響なのか，遺伝子多型に影響された飲酒による効果なのか区別することは難しい．この区別はたいへん重要である．予防という観点から，飲酒を勧めるべきかどうかに関わってくるからである．そこで，アルコール摂取による影響を除外した場合に*ALDH2*2*アレルを保有する人には何らかの変化があるかを厳密に調査することにした．長寿医療研究センター疫学研究部による大規模疫学調査(Shimokata, et al, 2000)で，地域住民から無作為に抽出した40歳代から70歳代の健常者約2,300名について血液検査，検尿，アルコール摂取量を含む生活・病歴調査などのメディカルチェックを実施し，*ALDH2*遺伝子多型との相関を調べた．その結果，*ALDH2*2*アレルを一つでも保有する女性は，アルコール摂取の影響を除外した場合でも血清中の過酸化脂質(LPO)量増加が有意に認められた．この結果は，ALDH2活性の低下がアルコール摂取とは無関係に酸化ストレスを増大させ，*ALDH2*2*アレルが加齢に伴う多くの疾患で危険因子となりうる可能性を示している(Ohsawa, et al, 2003a)．男性では有意差がなかったが，これは男性の場合はアルコール摂取によるLPO蓄積量への影響が強すぎる為であると予想される．では，ADでは*ALDH2*遺伝子多型がどのように影響するのかが問題となる．

4. *ALDH2*2*によるアルツハイマー病発症促進の分子機構

ここまで，*ALDH2*2*アレルの保有は，酸化ストレスを増大させてADの危険因子となることことが疫学調査により明らかとなったことを述べてきた．酸化ストレスの増大はアルコール代謝とは無関係に生じることから，ALDH2はアセトアルデヒド以外の基質を代謝することで酸化ストレスを抑制しているはずである．またALDH2はミトコンドリアにあることから，ミトコンドリアで生じるアルデヒド類を代謝しているはずである．では，そのアルデヒド類は何であろうか？

ミトコンドリア呼吸鎖から漏れた電子は酸素をスーパーオキシド・ラディカルとする．ここから，ヒドロキシラジカルなどの毒性の高いROSが派生し，これがカルディオリピンなどの不飽和脂肪酸を攻撃することでLPOが生じる．このLPOからは，酸化ストレスのマーカーとなるマロンジアルデヒドや毒性の強い4-ヒドロキシ-2-ノネナール(4-HNE)などのアルデヒド類が定常的に生じる．特に4-HNEはリジン，ヒスチジン，セリンおよびシステイン残基に容易に結合して蛋白の変性を引き起こす(Uchida & Stadtman, 1992)．実際，4-HNEはNa^+, K^+-ATPaseの活性を低下させ(Siems, et al, 1996)，*in vitro*において神経細胞死を促進することも示されている(Kruman & Mattson,

1999). さらにADやパーキンソン病などの神経変性疾患において4-HNEの蓄積が報告されている (Yoritaka, et al, 1996; Sayre, et al, 1997). 一方, 精製したALDH2は4-HNEを基質として酸化することができ, 細胞に4-HNEを添加するとアルデヒド脱水素酵素によって酸化されることにより生じる4-hydroxy-2-nonenoic acid (4-HNA) が検出された (Hartley, et al, 1995).

そこで, 筆者らは, ミトコンドリア酸化ストレスによって生じる4-HNEの除去にミトコンドリア酵素であるALDH2が関与しており, その活性が欠損すると酸化ストレスによる4-HNEの蓄積によって神経細胞死が促進されるはずであるという仮説を立てた. これを検証する為に, マウス・ラット型 *ALDH2*2* 遺伝子を作製し, これをラットPC12細胞に導入した. その結果, *ALDH2*2* 遺伝子を導入した細胞ではALDH2活性が抑制され, 4-HNEによって容易に細胞死が誘導された (図3). また, ミトコンドリア呼吸鎖の複合体III阻害剤であるアンチマイシンAによってROSを生じさせた場合もALDH2活性抑制細胞の細胞死が促進された. この時, ALDH2活性抑制細胞では4-HNEが高度に蓄積していた (図4). 以上の結果は上記の仮説を証明するものであり, ミトコンドリア酸化ストレス除去機構におけるALDH2の役割が細胞レベルで明らかとなった (Ohsawa, et al, 2003b).

5. ALDH2活性抑制トランスジェニック・マウスにおける中枢神経系の加齢に伴う変性

個体レベルで酸化ストレス防御における特定遺伝子の関与について解析するには, 遺伝子変異, ノックアウトあるいは遺伝子導入などのモデル動物育種が最良の方法の一つである. 実際にMnスーパーオキシドジスムターゼ (Mn-SOD) 欠損マウスでは酸化ストレスが蓄積し, ミトコンドリアの機能不全とそれに続く細胞死が観察される (Melov, et al, 1998). この結果は, 個体レベルでもMn-SODが酸化ストレス除去機構において最も重要な機能を果たしている酵素の一つであることを示しているが, このマウスは生後1週間程度で死んでしまい, 加齢に伴う変化を解析することは不可能である. 最近, ミトコンドリアのターゲット配列を付加したカタラーゼ遺伝子を導入したマウスでは寿命が延びることが報告された (Schriner, et al, 2005). この結果は, 老化における酸化ストレス制御の重要性を明確に示している.

ここでALDH2について考察してみよう. 前述のようにALDH2はアルデヒド脱水素酵素の大きなファミリーに属する. また, 細胞でALDH2により除去される4-HNEなどのアル

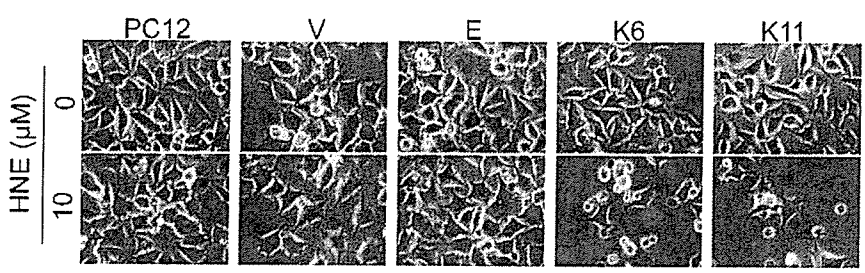

図3. 4-HNEに対して脆弱となった *ALDH2*2* 遺伝子導入PC12細胞
PC12細胞に *ALDH2*2* 遺伝子を導入してドミナント・ネガティブにALDH2活性を抑制した株 (K6, K11) では, ALDH2活性を保持する親株 (PC12), ベクター導入株 (V), *ALDH2*1* 遺伝子導入株 (E) では死なない濃度の4-HNEでも, 顕著な細胞死が見られた.

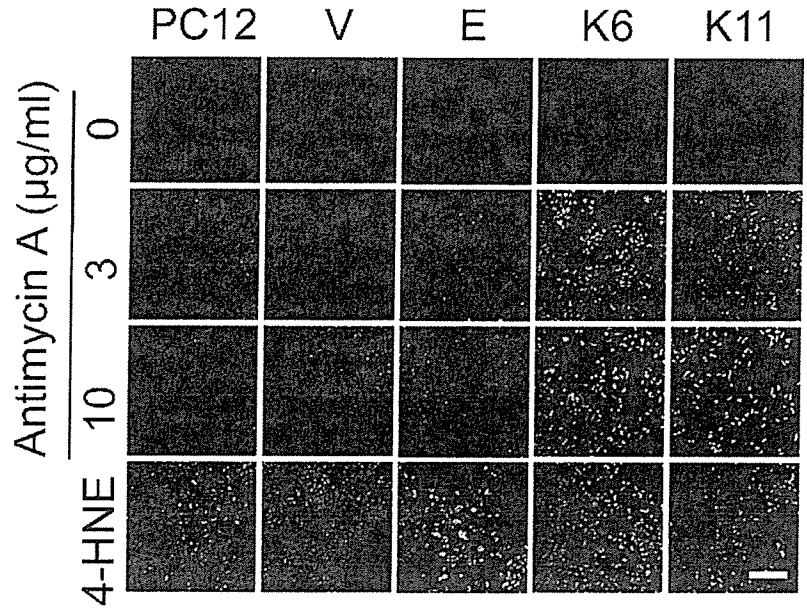

図4.　ALDH2活性の抑制による4-HNEの蓄積促進
呼吸鎖阻害剤アンチマイシンAを添加してROSを発生させ，24時間後の細胞内4-HNEをその特異的抗体で検出した．ALDH2活性を抑制した株では，顕著な4-HNEの蓄積が認められる．細胞株については，図3参照．

デヒド類に対しては，ALDH2に加えてグルタチオンなどの複数の解毒システムが細胞に存在する．こうした遺伝子の場合は，他の酵素などを代用することにより，ノックアウトしても大きな変化は見られない可能性が高い．事実，ALDH2ノックアウト・マウスではメトキシアセトアルデヒドの代謝低下は示されているが，発生過程や身体機能についての異常は見られない（Kitagawa, et al, 2000）．一方，人においては*ALDH2*2*アレルを持つ者はADの発症危険性が高いなど，ALDH2*2によるALDH2活性の抑制は，酸化ストレスの亢進が一因と考えられる種々の疾患の原因となる．そこで，モデル動物においても*ALDH2*2*遺伝子を導入し，ドミナント・ネガティブにALDH2活性を抑制することで，より人に近い状態のモデル動物が育種できるものと期待された．

まず，PC12細胞で用いたものと同じマウス型*ALDH2*2*遺伝子を汎用プロモーターであるEFプロモーター下に挿入し，これをマウスC57BL/6に導入することでトランスジェニック・マウスを作製した．作製したマウスはDAL（Dominant negative of ALDH2）マウスと命名した．このマウスはホモで維持しても発生過程での異常は認められず，雄についてのみ生後3ヶ月齢以降に全身で白髪が見られ，後肢の筋力低下が認められた（図5）．雌では24ヶ月齢まで観察を続けたが，C57BL/6と比較して身体所見に特に異常は認められなかった．そこで，PC12細胞と同様に4-HNEに対する脆弱性が中枢の神経細胞でも認められるか検討した．胎生16日のマウス胎児から大脳皮質を取り出し初代培養して，これに4-HNEを添加したところDALマウスでは神経細胞死が促進され（図6），脳における酸化ストレス亢進の可能性を示唆された．

それでは，DALマウスの脳で異常が認められるであろうか？　特にC57BL/6との差が認め

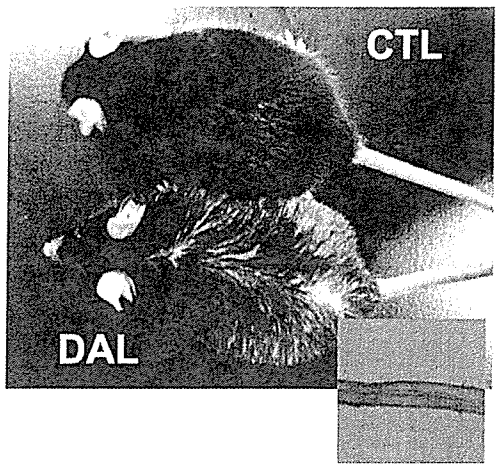

図 5.　DAL マウス
6ヶ月齢の DAL マウス雄．体毛に白髪が見られ，色素が抜けている(挿入写真)．CTL は同齢の C57BL/6 雄．

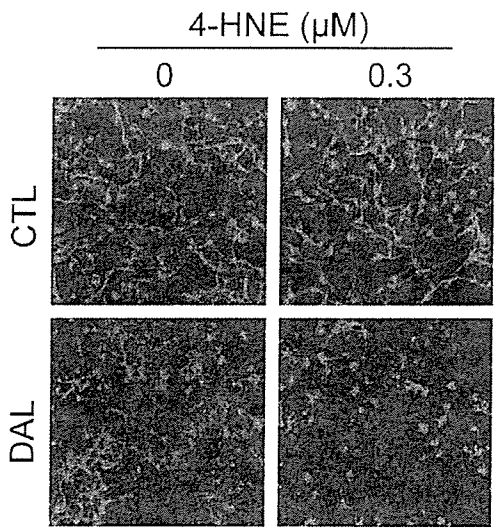

図 6.　4-HNE に対して脆弱となった DAL マウスの大脳皮質神経細胞．
脳で ALDH2*2 が発現している DAL マウス胎児大脳皮質から初代培養神経細胞を調製し，これに 4-HNE を添加して 24 時間後の細胞を固定，神経細胞特異的抗体である抗 TUJ-1 抗体により生存神経細胞を染色した．コントロール(CTL)の C57BL/6 より調製した神経細胞に比べ，DAL マウス神経細胞では低濃度の 4-HNE で細胞死が見られる．

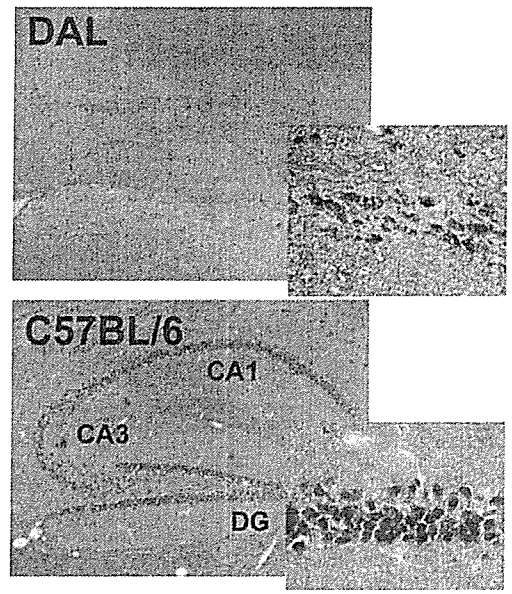

図 7.　DAL マウスにおける海馬の萎縮と錐体細胞の変性
DAL マウス(雌)では，18ヶ月齢で顕著な海馬の萎縮と錐体細胞の変性が認められる．HE 染色．挿入図は CA1 領域を拡大したもの．

られない雌について解析を行った．まず，6ヶ月齢のマウスについて脳の剖検を試みたが，C57BL/6 の脳と違いはなかった．しかし，18ヶ月齢の DAL マウスでは海馬の萎縮とそれに伴う錐体細胞の脱落やグリア細胞の活性化といった神経変性の所見が認められた (図 7)．こうした所見は 12ヶ月齢で散見されるようになり，加齢と共に増加する．しかし，運動機能や感覚機能については DAL マウスと C57BL/6 との間に顕著な差はなかった．そこで，海馬が関与する空間認知能力の試験として多用されている水迷路学習の課題を試みた．その結果の一部を図 8 に示す．DAL マウス 6ヶ月齢では，学習能力の低下は認められないが，18ヶ月齢では顕著な低下が見られた．こうした脳の変性と学習能力の低下は，初代培養神経細胞の場合と同様に酸化ストレスに対する抵抗性の低下によるものと考えられる．現在，加齢に伴う 4-HNE などの酸

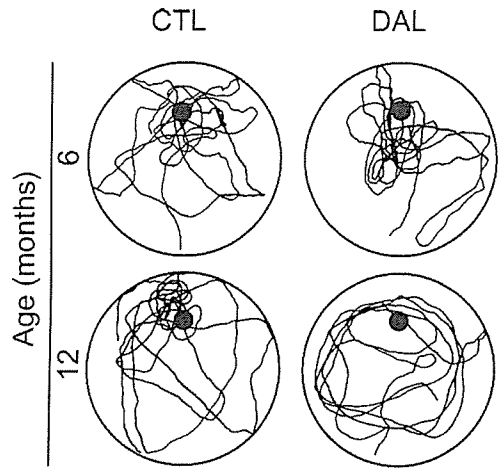

図8. 加齢に伴うDALマウスの学習能力低下
5日間の水迷路学習後にプラットフォームを除去し，60秒間のプローブテストを実施したときの泳路を示す．DALマウス(雌)は6ヶ月齢ではプラットフォームがあった位置（黒丸）を記憶してその周囲を遊泳したが，12ヶ月齢ではプラットフォームの位置に関係なくプール内を泳ぎ廻った．コントロール（CTL）は，C57BL/6雌．

化ストレス・マーカーの変化について解析中である．実際，筋特異的にALDH2*2を発現させた系統のDALマウスでは，筋萎縮の所見とそれに伴うミトコンドリア異常及び4-HNEの蓄積が認められている（Ohsawa, et al, 投稿準備中）．

6. ALDH2によって代謝される4-HNEとアルツハイマー病

今までの結果から，*ALDH2*2*アレルがアルツハイマー病の危険因子となるのは，加齢に伴う酸化ストレスの増加で脳に4-HNEなどの毒性の高いアルデヒド類が蓄積するが，これを除去する酵素の一つであるALDH2の活性が抑制されることでADの発症を促進している為である，と仮説を立てている(図9参照)．ここで，二つの疑問が生じる．第一にADの発症以前に4-HNEなどが蓄積するのであろうか？最近，軽度認知機能障害(MCI)および初期AD患者の海馬や中側頭回において，健常者に比して顕著な4-HNEの増加が報告された(Williams, et al, 2005)．これは，LPOの解析を脳髄液で行った結果(Pratico, et al, 2002)とも一致し，4-HNEに代表される酸化ストレスの蓄積がAD発症以前に起こっていると考えられる．第二に4-HNEなどの蓄積はADに特徴的な病変を引きおこすであろうか？ 4-HNEは神経細胞死を引き起こすだけでなく，Na^+, K^+ ATPase活性を低下させるなどの機構によりシナプスの機能低下をもたらし(Pedersen, et al, 1999)，微小管形成と神経突起進展を強く阻害する（Neely, et al, 1999)．さらにADの病理学的組織像に特徴的な神経原繊維変化（NFT）と酸化ストレスとの関わりについては多くの報告がなされている(Zarkovic, et al, 2003)．特に4-HNEに関しては，リン酸化タウを修飾することで構造変化を引き起こし，タウをNFTに存在する構造とすることが報告されており（Takeda, et al, 2000 ; Liu, et al, 2005)，NFTの形成に4-HNEが重要な役割を担っているものと考えられている．一方，老人班については，βアミロイド（Aβ）による酸化ストレスの亢進に関する報告は多数あるが，4-HNEとAβ産生機序との関わりについてはほとんど報告が無かった．しかし，最近になって4-HNEによるストレス応答経路の活性化でBACE1の発現量が上昇することが報告され，Aβ産生量を増加させている可能性が指摘されている（Tamagno, et al, 2005)．また，Aβ沈着モデルのトランスジェニック・マウスでは，Aβの蓄積前にLPOの蓄積が亢進されると報告されている（Pratico, et al, 2001)．

我々の疫学調査から，*ALDH2*2*アレルと*APOE ε4*アレルとの間で相乗的にADの発症リスクが増大していた．このAPOEと4-HNEとの関連については，AD患者脳を抗4-HNE抗体で免疫染色したところ錐体細胞における細胞質での陽性像が*APOE ε4*アレルをもつ者