Overexpression of Aldehyde Dehydrogenase-2 Attenuates Neurotoxicity Induced by 4-Hydroxynonenal in Cultured Primary Hippocampal Neurons

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Abstract 4-Hydroxynonenal (4HNE) is a toxic aldehyde which can accumulate during neurodegenerative diseases, such as AD. 4HNE-induced neuronal cytotoxicity includes the damage of neurite growth as well as a potential threat leading to the neuronal cell death. This study was designed to examine whether overexpression of aldehyde dehydrogenase-2 (ALDH2) affects 4HNE-induced neurite outgrowth blockage and neuronal death in primary hippocampal neurons in vitro. Plasmid-encoding rat ALDH2 was constructed and transfected into cultured rat hippocampal neurons. In vitro-cultured rat hippocampal neurons with the transfection of ALDH2 gene were showing resistance to 4HNE-induced neurite damage. Overexpression of ALDH2 in cultured rat hippocampal neurons blocked 4HNE-induced (3.2 μM for 24 h) reduction of neurite outgrowth and branching. In addition to the effect on neurite growth, ALDH2 overexpression also can protect neurons from 4HNE-evoked (10 μM for 24 h) neuronal death. Furthermore, we found that overexpression of ALDH2 can decrease the caspase-3 protein expression level; at the same time, it can decrease the reactive oxygen species (ROS) level and the disruption of mitochondrial transmembrane potential in cultured hippocampal neurons. Our data suggested that overexpressed ALDH2 gene may moderate 4HNE-induced neuronal death by regulating caspase-3 protein and ROS level in cultured hippocampal neurons. Based on these findings, ALDH2 gene can be a potential therapeutic target for treatment of neurodegenerative diseases, such as AD.

Introduction

Oxidative stress plays an essential role in the damages of neuronal structures and their functions. 4-Hydroxynonenal (4HNE) is a cytotoxic product of lipid peroxidation, levels of which are markedly increased in multiple neurological disorders, and cause functional impairments of the CNS. The increased level of 4HNE was found in oxidative stress-related degenerative diseases (Romero et al. 1998; Zarkovic 2003; Mattson 2009; Hyun et al. 2002). Furthermore, a significant increase in 4HNE has been detected in the ventricular fluid and brain of Alzheimer's disease (AD) patients and in the neurons of Parkinson's disease (PD) patients (Lovell et al. 1997; Selley 1998).

Extensive evidence indicates that 4HNE is implicated in the pathological changes in neurodegenerative diseases. 4HNE has been shown to block neurite outgrowth, disrupt neuronal microtubules, and affect cellular tubulin (Neely et al. 1999, 2005; Kokubo et al. 2008). In addition, neuronal microtubules are required for the development of functional neural networks. 4HNE also induces apoptotic cell death in PC12 cells and primary rat hippocampal neurons (Soh et al. 2000; Kruman et al. 1997). From this point of view, preventing 4HNE insults may protect the neurons during neurodegenerative progression.

Aldehyde dehydrogenase-2 (ALDH2) is a nuclear-encoded enzyme that is widely expressed in the CNS

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(Stewart et al. 1996; Zimatkin et al. 1992). ALDH2 is expressed in brain, but little is known about its functional role in neurons. Recently, it has been suggested that age-dependent neurodegeneration accompanies memory loss in ALDH2-deficient mice (Ohsawa et al. 2008). Moreover, a recent study has indicated that ALDH2 as an enzyme whose activation correlates with reduced ischemic heart damage in rodent models, probably for the removal of 4HNE (Chen et al. 2008). Based on the studies, it raises the possibility that ALDH2 may play a role in the cellular defenses against neurotoxic effects induced by 4HNE. However, few data exist regarding the protective value of ALDH2 on 4HNE-induced neurite damage and the subsequent apoptosis in neurons, and the possible mechanisms.

Therefore, we hypothesize that overexpression of ALDH2 should accelerate the removal and detoxification of the toxin 4HNE and thus alleviate neuronal death and neurite damage resulting from 4HNE exposure. In order to learn more about ALDH2 and its protective functions, we upregulate the ALDH2 expression in hippicampal neurons. Our data indicated that ALDH2 protected neurons from 4HNE-mediated neurotoxic effects. The results of this study demonstrated that exposure to 3.2 μM 4HNE for 24 h resulted in an inhibition of neurite outgrowth of cultured hippocampal neurons, and upregulated ALDH2 expression blocked the 4HNE-induced decrease in neurite outgrowth. The results also demonstrated that exposure to 10 μM 4HNE for 24 h resulted in an increase in apoptotic cell death of cultured hippocampal neurons, and upregulated ALDH2 expression suppressed the 4HNE-induced neuronal death, reduced cleaved caspase-3 levels, and decreased intracellular ROS levels.

These results not only suggested the role of 4HNE in neuronal damage through a dose-dependent mechanism, but also for the first time provided convincing evidence that overexpressing ALDH2 may be a useful tool for the prevention and treatment of 4HNE-induced neuronal injury. This study also offered novel insights into the mechanism underlying how ALDH2 moderates 4HNE-induced neuronal death. Furthermore, studies indicated that the ALDH2 deficiency is a risk factor for late-onset AD in the Chinese and Japanese population (Wang et al. 2008; Kamino et al. 2000). Thus, our results reveal an important neuroprotective function of ALDH2 under 4HNE-exposure conditions, and suggest ALDH2 may be a key therapeutic or preventive target for the treatment of neurodegenerative diseases, such as AD.

Materials and Methods

Primary Hippocampal Neurons Culture

Hippocampus tissue was obtained from embryonic day 18 Sprague-Dawley rats. Protocol for disassociation of neurons from hippocampus was described in previous literature (Chen et al. 2006). In brief, hippocampus was removed from the brain under sterile condition and minced into 0.2–0.5 cm size. Then the tissue was digested by 0.125% (w/v) trypsin (Invitrogen, Carlsbad, CA, USA) for 20 min at 37°C. Disassociated neurons were collected by centrifugation at $1,000 \times g$ for 10 min. Primary hippocampal neurons were plated and maintained in Neurobasal A media (Invitrogen) supplemented with B27 supplement and 0.5 mM L-glutamine (all from Invitrogen) in 24-well plates coated with 50 μg/ml poly-D-lysine (Sigma-Aldrich, USA). Cells were incubated at 37°C, 5% CO_2 in a humidified cell culture incubator. Media was changed every 3–4 days. Primary neurons were selected for experiments after maintaining in culture for 7 days (DIV).

Plasmid Construct and Transfection

For rat ALDH2 plasmid construction, PCR products for ALDH2 were amplified by RT-PCR from rat brain total mRNA using the RT-PCR system (Promega, Madison, WI, USA) with the following primers: sense, 5′-GCCGCAGAC CGTGGTTACTT-3′; antisense, 5′-GCCGCTCCCCGACA TCTTAT-3′.

Then, the PCR products were gel purified and cloned into the pMD18-T vector (TaKaRa, Japan) following manufacture's instructions. In order to generate ALDH2 expression constructs, the resulting rat ALDH2 cDNA fragment was subcloned into mammalian expression vectors pEGFP-N2 (Clontech Laboratories, CA, USA). After 7 days, neurons were transfected with electroporated with a nucleofection kit for rat neurons (Amaxa, Gaithersburg, MD, USA) according to the supplier's instructions. All neurons were used for experiments 48 h after transfection.

Quantitative RT-PCR Analysis

Total RNA from cultured neurons was prepared using SV Total RNA Isolation System (Promega). For RT-PCR analysis, 2 μg of RNA was transcribed into cDNA with the RT-PCR System (Promega) in 20 μl reaction volume. The cDNA was then analyzed in a PCR assay using the primers as used in plasmid construction. Amplification reactions were performed using 0.2 μM of each primer, 1 μl cDNA and Taq DNA polymerase (Promega). PCR products were separated by electrophoresis in 1.75–2% agarose gels and were visualized by ethidium bromide staining.

Western Blot Analysis

Expression and secretion of ALDH2 protein by neurons following transfection was verified by Western blot of conditioned media and cellular lysates. Total protein

extracts were prepared from neurons after transfection. Equal amounts of total protein were separated on 12% sodium dodecyl sulfate–polyacrylamide gels and electrophoretically transferred to polyvinylidene difluorid membrane. The membrane was probed with primary antibodies against ALDH2 protein (Santa Cruz Biotechnology, CA, USA) or β-actin and sequentially incubated with secondary antibodies. The identified proteins were quantified by measuring optical densities of the bands.

Immunocytochemistry and Antibodies

Neuronal outgrowth was evaluated by immunocytochemistry assay after treatment with 4HNE for 24 h. In order to identify neuronal morphogenesis, cultured hippocampal neurons were immunostained with Anti-MAP2 antibody (Invitrogen; at 1:100 dilution). Neurons cultured on coverslips were fixed by replacing medium with a solution of 4% paraformaldehyde in PBS and washed in PBS. Subsequently, cells were blocked with 3% goat serum in PBST (PBS plus 0.3% Triton X-100) for 2 h at room temperature. Anti-MAP2 antibody was added and incubated overnight at 4°C and incubated with secondary fluorescent antibodies (Invitrogen).

Measurement of Neurite Outgrowth and Branching

Images were taken on a confocal laser-scanning microscope. Neurons were examined for neurite length measurements by tracing the total length of any neurite extending from a neuron cell body. Neurite outgrowth was observed in 30 randomly selected neurons per coverslip and analyzed by Axiovision Software (Carl Zeiss, Germany). The numbers of primary neuritis emerging directly from N somata were counted and from each neuritic tree, the total number of branches recorded using Axiovision software (Carl Zeiss, Germany).

Assess of Cell Death and Apoptosis

Cell viability was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetry assay after treatment with 4HNE for 24 h. In order to measure the mitochondrial function of surviving cells, MTT was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/ml as a 100-fold stock solution. Cell viability was estimated by the proportion of unstained cells. For morphological determination of apoptosis, hippocampal neurons were grown in 6-well plates, and the nuclei were stained with Hoechst 33258 (Sigma). The cells were then stained with Hoechst 33258 for 10 min at room temperature and covered with glycerol. Nuclei were visualized using an Olympus IX70 inverted fluorescence microscope.

Assess of Caspase-3 Activity

The caspase-3 activity was measured using an Apo-ONE Homogeneous Caspase-3 Assay Kit (Promega), according to the manufacturer's instructions. In brief, neurons were rinsed twice with PBS and collected in a tube. The cells were lysed in 50 μl of Homogeneous Caspase-3 Buffer containing the caspase-3 substrate, Z-DEVD-rhodamine 110, and the cell lysates were incubated for 14 h at room temperature. After incubation, the cleaved caspase-3 fluorescent images were analyzed with an Olympus IX70 inverted fluorescence microscope.

Assess of ROS Level and Mitochondrial Transmembrane Potential

The fluorescent dyes CM-H2DCFDA (Molecular Probe) were used to estimate total level of ROS production in neurons. Neurons were loaded with CM-H2DCFDA. The ROS production was determined by measuring the fluorescence. Then, the cells were incubated with 1 mM DCF-DA for 1 h at 37°C and were washed three times with PBS. After centrifugation at $1,000 \times g$ for 5 min, the supernatants were removed, and the pellets were resolved with 1% Triton X-100, and fluorescence was measured by a fluorescence microplate reader (excitation wavelength of 480 nm and an emission wavelength of 540 nm). It has been shown that the uptake of rhodamine 123 into mitochondria is a function of mitochondrial transmembrane potential. Neurons were incubated for 20 min at 37°C in DMEM containing 10 μM rhodamine 123. Neurons were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 510 nm using a fluorescence microplate reader. Appropriate positive and negative controls were included in each measurement.

Statistical Analysis

Data in text and figures are expressed as mean ± SE. Two group comparisons were evaluated by paired or unpaired t-tests, as appropriate. Multiple comparisons were analyzed by ANOVA and Tukey's or Newman Keuls post hoc tests. A value of $P < 0.05$ was considered significant.

Results

Transient ALDH2 Transfection Induces Overexpression of ALDH2 mRNA and Protein in Hippocampal Neurons

ALDH2 overexpression was induced by transfections with ALDH2 plasmid cDNA. Before transfection, we examined

the expression of NSE in primary cultured hippocampal neurons by immunofluorescence staining and found 90% of cells as neurons (data not shown). Primary cultured hippocampal neurons at 7DIV were transfected with ALDH2 cDNA or control vector for 48 h. Laser scanning confocal microscopy of neurons transfected with expressing vectors revealed a transfection efficiency between 25 and 50% (data not shown), so electroporated transfection was effective.

We first measured the expression of ALDH2 mRNA neurons after transfection by quantitative RT-PCR, which was significantly increased compared with control group (Fig. 1a). ALDH2 protein expression was determined by
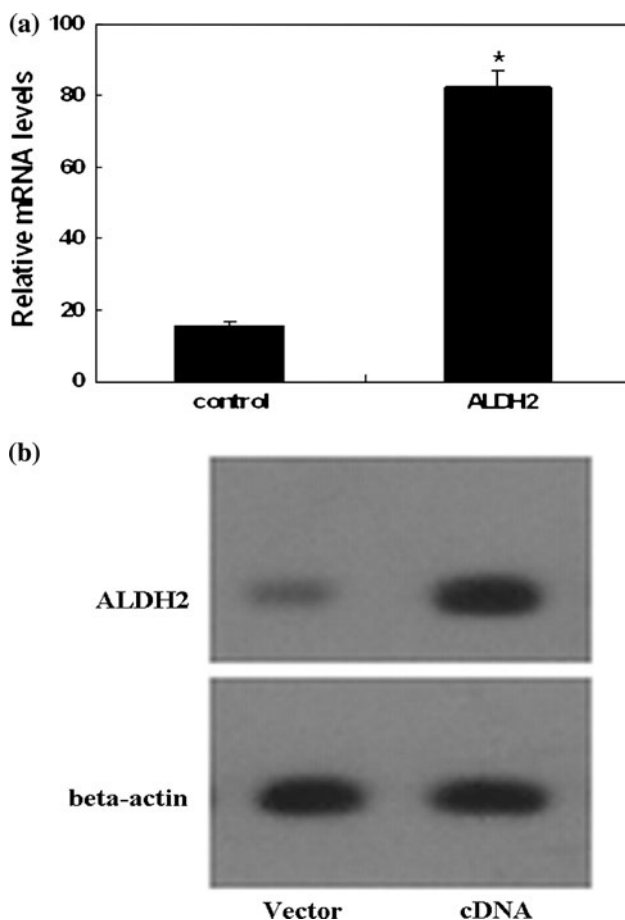
Fig. 1 ALDH2 mRNA and protein were expressed in DIV7 hippocampal neurons following transfection. Neurons were transfected with 1.0 μg/40 neurons of ALDH2, full-length ALDH2 or vector for 48 h. Following the transfection, ALDH2 mRNA and protein expressions were determined by quantitative PT-PCR and western blotting, respectively. **a** ALDH2 mRNA expression after transfection. Data were expressed as fold increase over vector transfection ($n = 6$ per group, $* P < 0.01$ vs. vector). **b** Western blots of ALDH2 protein expression following transfection ($n = 6$ per group, $P < 0.01$ vs. vector)

western blot, which was also elevated following transfection as compared with control group (Fig. 1b).

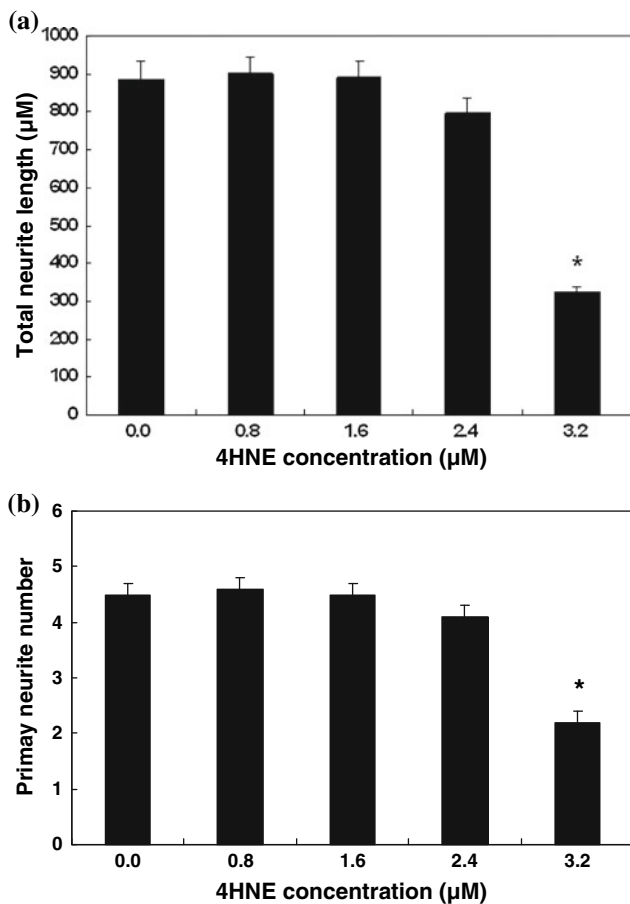## 4HNE Inhibits Neurite Outgrowth in Cultured Hippocampal Neurons

Previous studies in neuronal cell cultures have shown that the 4HNE induces the inhibition of neurite outgrowth with increasing concentrations of 4HNE (Neely et al. 1999). In this study, we examined whether 4HNE can block neurite outgrowth in primary cultured hippocampal neurons with increasing concentrations. Thus, before studying the relationship between 4HNE-induced neurite toxicity and ALDH2 overexpression, we exposed primary cultures of rat hippocampal neurons to different concentrations of 4HNE. We investigated the neurite inhibitory action of 4HNE using the hippocampal neurons culture at 7DIV, because the DIV7 cultures have generated lots of long neurites.

In order to study the inhibitory effect of 4HNE on neuronal outgrowth, hippocampal neurons in vitro were treated with 0.8–3.2 μM 4HNE for 24 h. Immunostaining with anti-MAP2 antibody revealed that the MAP2-positive neurites derived from neurons were significantly decreased by the treatment with 3.2 μM 4HNE. They had shorter neurites, smaller primary neurite numbers and decreased branching frequency than those under the normal condition. The total neurite length significantly fell from 888.6 ± 51.2 μm in neurons treated with vehicle to 321.8 ± 10.5 μm in neurons after 24 h 4HNE treatment ($P < 0.01$, Fig. 2a). Similarly, the number of primary neuritis significantly fell from 4.5 ± 1.2 in neurons of control to 2.2 ± 0.5 in neurons after 24 h 4HNE treatment ($P < 0.01$, Fig. 2b). Cell viability was analyzed at the time that neurites were measured. No significant cell death was observed in any of the cultures (data not shown).

ALDH2 Protects Hippocampal Neurons Against 4HNE-Induced Neurite Damage

In order to confirm the neuroprotective effect of ALDH2, we sought to examine whether overexpression of ALDH2 could protect neurons against 4HNE-induced neurite toxicity. To this end, we transfected rat neurons at 7DIV with control vector (Fig. 3a) or ALDH2 expressing vector (Fig. 3b), and we could see that these transfected neurons allowed the majority of neurons to extend up to four or five primary neuritis (Fig. 3a, b). Then, we treated the transfected neurons with 3.2 μM 4HNE accordingly.

Consistent with the inhibition of neurite outgrowth in primary cultured neurons, the length of neurites was significantly reduced after 24 h incubation of 4HNE (3.2 μM) in the neurons transfected with vector only (Fig. 3c). They

Fig. 2 Statistically significant neurite inhibition was observed at 3.2 μm HNE. Neurons at 7DIV were added with varying concentrations of HNE for 24 h, and neurite outgrowth was measured after incubation. Cultures were fixed and immunostained for the neurite-selective antigen MAP2. However, exposure to 3.2 μM HNE significantly reduced the length of the neurite and decreased the primary neurite number. **a** The effect observed after treatment with 0–2.4 μM HNE was not statistically significant; it showed that exposure to 3.2 μM HNE significantly reduced the neurite length. **b** Exposure to 3.2 μM HNE significantly reduced the primary neurite number. Values are the mean ± SE of at least three independent experiments. * $P < 0.01$, significantly different from control values

had shorter neurites and smaller primary neurite numbers than those without addition of 4HNE (Fig. 3a). However, neurite length was restored in neurons expressing a plasmid expressing full-length ALDH2 when exposed to 3.2 μM 4HNE (Fig. 3d), indicating protection against 4HNE-induced neurite toxicity.

When compared with those vector alone-expressing neurons under 4HNE treatment, ALDH2 blocked the inhibitory effects of 4HNE on neurite outgrowth (primary neurite number: 3.6 ± 1.1; $P < 0.05$; total neurite length: 566.6 ± 15.7 μm; $P < 0.05$) (Fig. 3e, f). These results, together with previous results, provide evidence that ALDH2 protected neurons from 4HNE-mediated inhibition of neurite outgrowth and neurite loss.

4HNE Induces Neuronal Death in Cultured Hippocampal Neurons

Previous studies in primary neuronal cultures have shown that the 4HNE induces the death of CNS neurons in a concentration-dependent manner (Peng et al. 2007).

In this study, we examined whether 4HNE can cause neuronal death in primary cultured hippocampal neurons in a concentration-dependent manner. Thus, in order to study the relationship between 4HNE neurotoxicity and ALDH2 expression, we exposed primary cultures of rat hippocampal neurons to different concentrations of 4HNE (2–10 μM) for 24 h. The MTT assay indicated a significant decrease (50%) in cell viability with 10 μM HNE treatment (Fig. 4).

## Overexpression of ALDH2 Decreases Neuronal Death Induced with 4HNE

In order to examine whether overexpression of ALDH2 could improve cell viability, cellular responses after 4HNE treatment were assessed using MTT assay.

Primary cultured hippocampal neurons at 7DIV were transfected with ALDH2 DNA or vector for 48 h, respectively. In neurons transfected with vector, 4HNE treatment significantly decreased cell survival. The percentage of cell survival assessed by MTT significantly fell from 84.6 ± 12.1% in vector-neurons without 4HNE to 42.6 ± 9.3% in vector-neurons after 24 h 4HNE treatment ($P < 0.01$, Fig. 5). The percentage of cell survival assessed by MTT increased (68.7 ± 8.9%; $P < 0.05$, Fig. 5) in ALDH2-neurons after 24-h 4HNE treatment when compared with vector-neurons after 24-h 4HNE treatment. Thus, ALDH2 overexpression improves overall cell survival.

4HNE Induces Apoptotic Neuronal Death in cultured Hippocampal Neurons

In order to examine whether 4HNE-induced cell death was apoptotic cell death, we used Hoechst staining to identify the neuronal apoptosis. Results derived from the Hoechst staining assays showed similar findings (Fig. 6a). Incubation of hippocampal cultures with 4HNE caused dose-dependent cell death. The percentage of apoptotic cells was almost 50% when neurons were treated with 10 μM 4HNE. Therefore, all the subsequent experiments were conducted with 10 μM 4HNE.

Activation of caspase-3 has been widely regarded as a major mechanism in neuronal apoptosis. In addition, previous studies have shown that 4HNE can induce neuronal apoptosis by a mechanism involving activation of caspase-3 (Camandola et al. 2000). In order to determine whether the neuronal apoptosis induced by 4HNE was associated with activation of caspase-3, we also performed
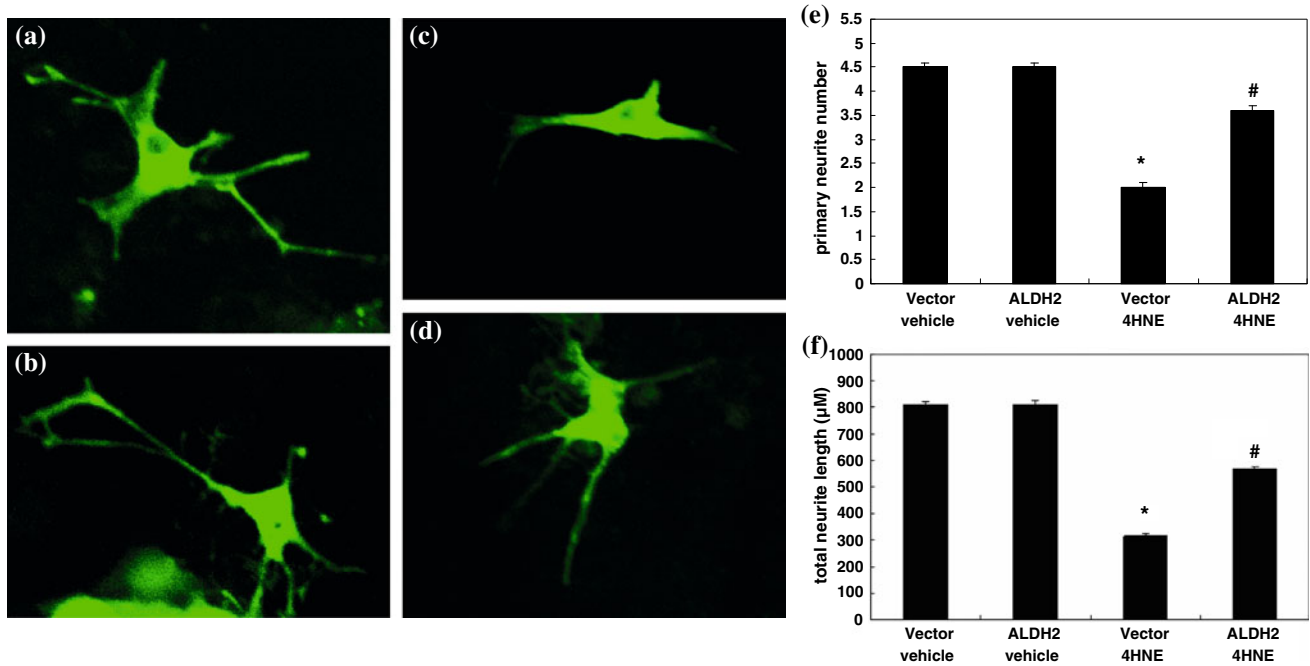
Fig. 3 ALDH2 overexpression prevents the reduction in neurite outgrowth evoked by 4HNE. Neuronal cultures at 7DIV were transfected with either ALDH2 or the control vector with extended neuritis, as described in "Materials and Methods" section. **a** Neuronal cultures at 7DIV were transfected with control vector. **b** Neuronal cultures at 7DIV were transfected with ALDH2 cDNA. Approximately 48 h later, transfected neurons were treated with 3.2 μM 4HNE. **c** Neurons with vector only exposed to 3.2 μM 4HNE significantly inhibited neurite outgrowth and decreased neurite number. **d** ALDH2 overexpression blocks the reduction in neurite outgrowth evoked by 4HNE. Photomicrographs were obtained using a laser-scanning confocal microscope. Scale bar = 20 μm. The figures are representative for three different experiments. **e** Primary neurite number was restored in neurons expressing ALDH2-cDNA when compared with those vector alone expressing neurons under 4HNE treatment. **f** Neurite length was restored in neurons expressing ALDH2-cDNA when compared with those vector alone-expressing neurons under 4HNE treatment. Values are the mean ± SE of at least three independent experiments. * $P < 0.01$ versus neurons expressing vector under normal (vehicle) condition. # $P < 0.05$ versus neurons expressing vector under 4HNE-treated condition
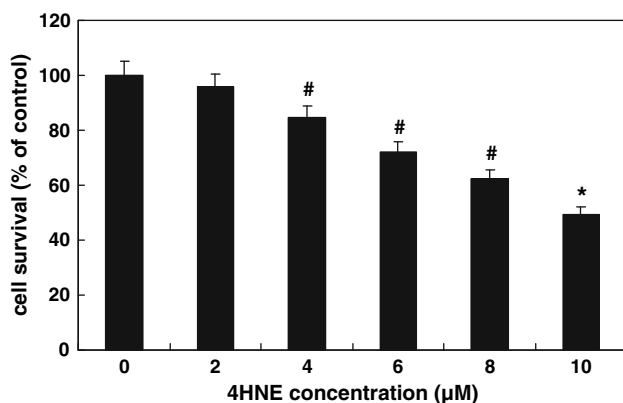
Fig. 4 4HNE induces neuronal death. Neuronal cultures were treated with different concentrations of 4HNE. 4HNE decreased the cell survival in a dose-dependent manner. Cell survival was measured with MTT 24 h after treatment with 4HNE. It showed that exposure to 10 μM 4HNE significantly reduced cell survival. Values are the mean ± SE of at least three independent experiments. # $P < 0.05$, * $P < 0.01$, significantly different from control values
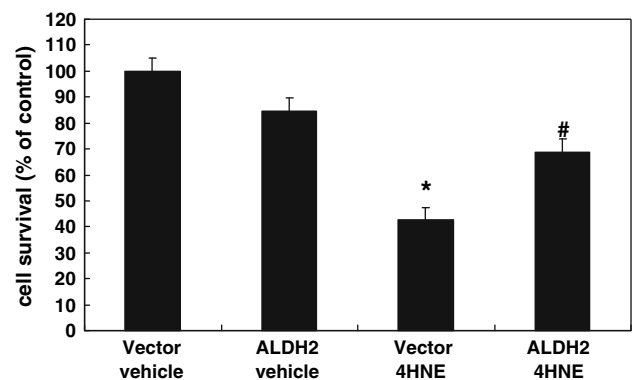
Fig. 5 The neuroprotective effects of ALDH2 on 4HNE-induced neuronal death. Neuronal cultures were transfected with either ALDH2 or the control vector, as described in "Materials and Methods". Approximately 48 h later, neurons were treated with 10 μM 4HNE. Cell survival was measured with MTT 24 h after treatment. Values are the mean ± SE of at least three independent experiments. * $P < 0.01$ versus neurons expressing vector under normal (vehicle) condition. # $P < 0.05$ versus neurons expressing vector under 4HNE-treated condition

(a)

(b)
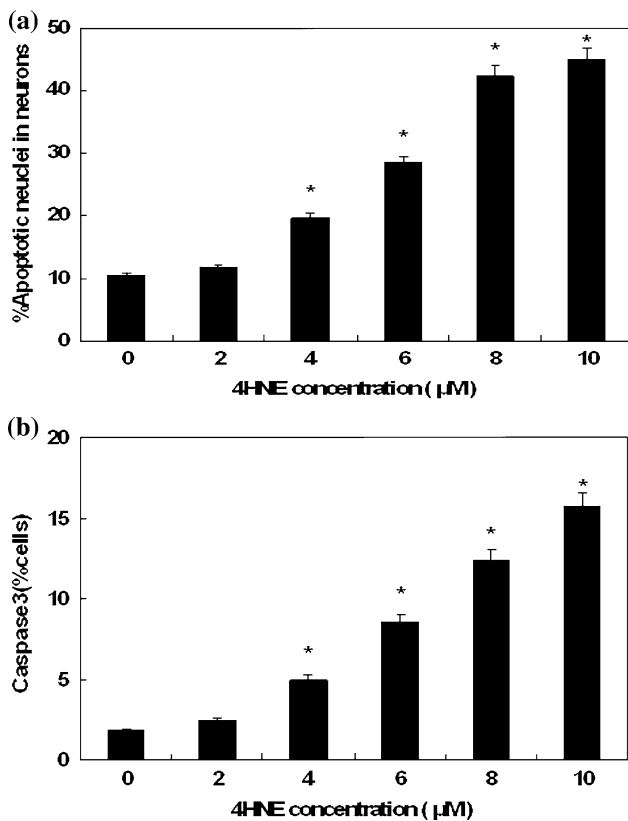
Fig. 6 4HNE induces apoptotic neuronal death. Neuronal cultures were treated with 10 μM of 4HNE. **a** Apoptosis alterations of hippocampal neurons after 24 h of vehicle (control) or 4HNE treatment were measured with nuclear Hoechst 33258 staining and fluorescence assessment. **b** Assessment of cleaved caspase-3 alterations. It showed that exposure to 10 μM 4HNE significantly increased neuronal apoptosis. Values are the mean ± SE of at least three independent experiments. * $P < 0.01$, significantly different from control values

immunocytochemistry studies using an antibody that recognizes only the active form of this enzyme. 4HNE (10 μM) induced an increase of caspase-3 in the percentage of neurons (Fig. 6b). We did not observe alterations of caspase-9 activation after 4HNE treatment in cultured neurons (data not shown).

ALDH2 Overexpression Rescues Hippocampal Neurons from Apoptosis Evoked by 4HNE

In order to further confirm the neuroprotective effect of ALDH2, we sought to examine whether overexpression of ALDH2 could protect neurons against 4HNE-induced apoptotic cell death. Similarly, primary cultured hippocampal neurons at 7DIV were transfected with ALDH2 DNA or control vector. In order to examine whether overexpression of ALDH2 could attenuate 4HNE-induced apoptosis, cellular responses after 4HNE treatment were assessed using caspase-3 activity.
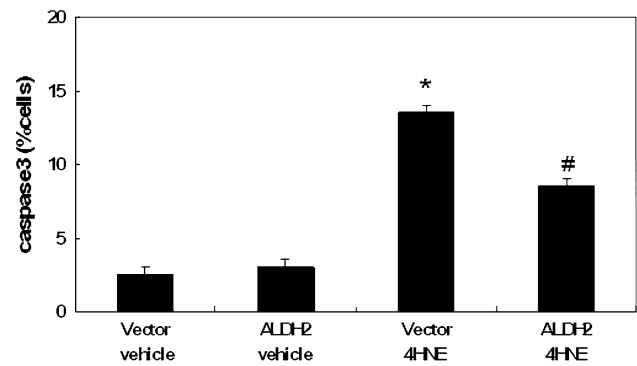
Fig. 7 The neuroprotective effects of ALDH2 on 4HNE-induced neuronal apoptosis. Neuronal cultures were transfected with either ALDH2 or the control vector, as described in "Materials and Methods". Approximately 48 h later, neurons were treated with 10 μM 4HNE. The percentage of neurons with cleaved caspase-3 was measured by immunocytochemistry using a cleaved caspase-3 polyclonal antiserum 48 h after treatment with 4HNE. Values are the mean ± SE of at least three independent experiments. * $P < 0.01$ versus neurons expressing vector under normal (vehicle) condition. [#] $P < 0.05$ versus neurons expressing vector under 4HNE-treated condition

In neurons transfected with vector, only 4HNE treatment significantly increased caspase-3 activity (Fig. 7). The percentage of caspase-3 expression significantly increased from $2.5 \pm 1.1\%$ in vector-neurons without 4HNE to $13.5 \pm 1.3\%$ in vector-neurons after 24 h 4HNE treatment ($P < 0.01$, Fig. 7). The percentage of caspase-3 expression decreased ($8.5 \pm 1.9\%$; $P < 0.05$, Fig. 7) in ALDH2-neurons after 24-h 4HNE treatment when compared with vector-neurons after 24-h 4HNE treatment. Thus, ALDH2 overexpression has decreased 4HNE-induced apoptosis.

Effect of ALDH2 on the Loss of Mitochondrial Transmembrane Potential Caused by 4HNE

The collapse of mitochondrial transmembrane potential has been considered as the early phenomena in the apoptotic process (Chandra et al. 2000). Therefore, we examined the effect of 4HNE on the mitochondrial transmembrane potential. Change in the mitochondrial transmembrane potential in neurons treated with 4HNE was quantified by measuring the cellular retention of rhodamine 123. When neurons were treated with 10 μM 4HNE for 24 h, a decrease in the retention of rhodamine 123 was observed. However, ALDH2 prevented the 4HNE-induced decrease in the retention of rhodamine 123 (Fig. 8).

Effect of ALDH2 on 4HNE-Induced Increase in Intracellular Reactive Oxygen Species Level

4-Hydroxynonenal has been suggested to be a key mediator of oxidative stress-induced cell death and to induce mitochondrial oxidative stress (Poli and Schaur 2000; Raza and
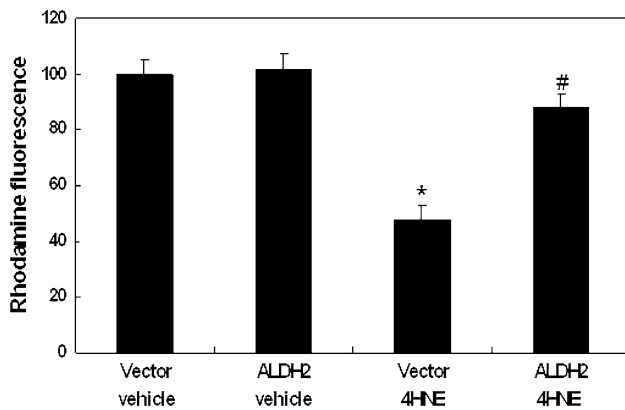
Fig. 8 Effect of ALDH2 on the loss of mitochondrial transmembrane potential caused by 4HNE Mitochondrial membrane potential alteration was measured by fluorescence microplate reader using rhodamine 123 staining, after vector or ALDH2 expressing neurons were exposed to vehicle or 10 μM 4HNE for 24 h. Values are the mean ± SE of at least three independent experiments. * $P < 0.01$ versus neurons expressing vector under normal (vehicle) condition. # $P < 0.01$ versus neurons expressing vector under 4HNE-treated condition

John 2006). In order to examine whether the inhibitory effect of ALDH2 on the toxicity of 4HNE is mediated by decreasing ROS level, neurons were treated with 10 μM 4HNE for 24 h, and the levels of ROS were measured using DCF fluorescence. When neurons were exposed to 4HNE, the intracellular ROS level significantly increased from 48.9 ± 5.9% (control) to 158.9 ± 11.3% (10 μM 4HNE, $P < 0.01$), revealing that 4HNE-induced intracellular ROS level in neurons. However, neurons transfected with ALDH2 effectively reduced ROS generation. ALDH2 inhibited the 10 μM 4HNE-induced increase in DCF fluorescence (Fig. 9).
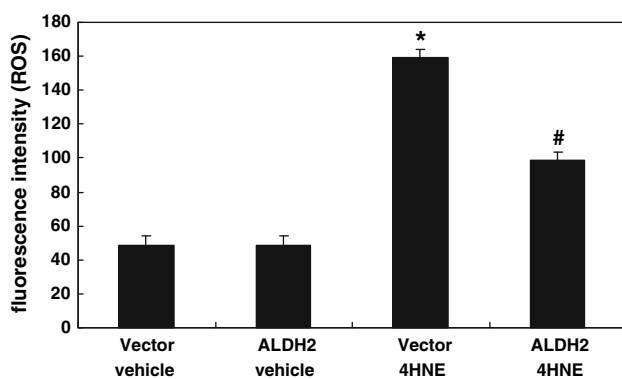
Fig. 9 ALDH2 overexpression lowers intracellular and mitochondria-derived ROS production induced by 4HNE. Intracellular ROS levels were measured by fluorescence intensity of DCF, after vector or ALDH2 expressing neurons were exposed to vehicle or 10 μM 4HNE for 24 h. Values are the mean ± SE of at least three independent experiments. * $P < 0.01$ versus neurons expressing vector under normal (vehicle) condition. # $P < 0.05$ versus neurons expressing vector under 4HNE-treated condition

Discussion

In our study, ALDH2 exerts a neuroprotective effect in rat primary cultured neurons. These results not only suggested a role of 4HNE in neuronal damage through a dose-dependent mechanism but also provided convincing evidence that ALDH2 may be a potential therapeutic tool for the prevention and treatment of 4HNE-induced neuronal injury. Upregulation of ALDH2 expression with transfection prevents the neurite damage and the apoptotic neuronal death evoked by 4HNE. Together with the observation, we can conclude that transgene overexpression of ALDH2 reduces these neurotoxic effects of 4HNE and contributes to neuroprotection.

In this article, we report a novel neuroprotective effect of ALDH2, which involves the 4HNE, a toxin implicated in neurodegeneration that, at the same time, has been associated with various CNS disorders. 4HNE are implicated in several neurodegenerative disorders including AD (Keller and Mattson 1998), ALS (Pedersen et al. 1998) and PD (Yoritaka et al. 1996). Concentrations of free 4HNE are elevated in the CSF (1.47 ± 0.76 vs. 0.38 ± 0.14 μM) of patients with PD compared with controls (Selley 1998). In addition, it has been reported that 4HNE concentrations in the range of those found in PD CSF are toxic to DA neurons in rat mesencephalic cultures (Selley 1998). Furthermore, 4HNE concentrations ranged from 0.68 to 181.1 μM in CSF from AD patients have been reported (Lovell et al. 1997). Previous studies have proved that 4HNE is found to be toxic to cerebral cortical neurons and oligodendrocytes at concentrations comparable to those measured in the CSF of patients with AD (Long et al. 2008; McCracken et al. 2000). In our study, 4HNE causes neurotoxic effects in hippocampal neurons at concentrations also within those measured in AD CSF.

Alzheimer's disease is a progressive neurodegenerative disorder that is the most common cause of dementia in the elderly. Deposition of aggregated amyloid beta-protein (Aβ) in neuritic plaques is the hallmark of AD. Data from studies of AD patients and experimental models suggest that Aβ damages neurons by causing the production of the lipid peroxidation product 4HNE (Kruman et al. 1997; Mark et al. 1997; Sayre et al. 1997). During neurodegenerative diseases, AD is characterized with memory disturbances and other cognitive symptoms. The brain reserve capacity is determined by the number of neurons and their synaptic and dendritic arborisation. Therefore, AD can be linked to a decreased reserve capacity of the brain, including reduced neuron number, decreased synaptic, and dendritic arborization. The characteristic events of the neurodegenerative process in AD commence with the early loss of synaptic contacts, then neurite damage, neuronal shrinkage, and, ultimately lead to neuronal degeneration and dementia (Klein 2002; Walsh and Selkoe 2004).

Our data not only offered support to neurite damage as an early and pivotal event in 4HNE deposition, but also provided evidence showing that progressive 4HNE deposition caused neuronal loss. We have confirmed that 4HNE does indeed induce a concentration-dependent reduction in neurite outgrowth and branching and neuronal survival in primary cultures of neurons. Preconditioning by pretreatment with 10 μM 4HNE for 24 h and pretreatment with 3.2 μM 4HNE for 24 h separately caused neurotoxic and neurite-toxic insults in hippocampal neurons. Our data are in agreement with the neurotoxic and neurite-toxic actions of 4HNE in various neuronal culture models (Neely et al. 1999; Rabacchi et al. 2004; Tang et al. 2008; Floyd and Hensley 2002). Moreover, despite intense research, it remains unclear how 4HNE interacts with neurons and triggers the biochemical signaling cascade that leads to neuronal dysfunction and neurotoxicity.

ALDH2 converts acetaldehyde to acetic acid. ALDH2 is a key enzyme in ethanol metabolism and involved in detoxification of aldehyde. It has been reported previously that deficiency of ALDH2 increases cell susceptibility to oxidative stress, and it also increases the risks in the development of AD (Ohsawa et al. 2008). Overexpression of ALDH2 may detoxify acetaldehyde and prevent 4HNE-induced cell injury in human umbilical vein endothelial cells (Li et al. 2004). mtALDH overexpression also attenuates hyperoxia-induced cell death in lung epithelial cells through reduction of ROS, activation of cell survival signaling pathways (Xu et al. 2006). Moreover, activation of ALDH2 can protect heart from ischemic damage (chen et al. 2008). ALDH2 is widely expressed in the CNS neurons (Stewart et al. 1996; Zimatkin et al. 1992). Despite these findings, not much is known about the exact functions of ALDH2 in primary cultured neurons.

We proved that overexpression of ALDH2 with transfection prevents both the neurite damage and the neuronal apoptosis evoked by 4HNE. ALDH2 effectively protected hippocampal and hippocampal neurons against 4HNE neurotoxicity. Therefore, removal of 4HNE through upregulating ALDH2 might play an important role in neurotoxicity leading to aged neuronal pathology. Our hippocampal neuron cultures at DIV7 are the perfect tool to study neuronal outgrowth. Hippocampal neurons allow us to study the inhibitory effects of 4HNE on neurite outgrowth, because the DIV7 cultures have generated lots of long neurites. We show here that 4HNE, a product of lipid peroxidation that accumulates in AD neurons, inhibits neurite growth. This neurotoxicity was not observed until 3.2 μM after 4HNE treatment, and when lower concentrations was analyzed, the neurite length of hippocampal cells appeared to be unaffected during the first 0.8–2.4 μM of treatment. It probably shows that certain concentration of 4HNE has neurite toxicity. Neurons have their polar shape. Neurons

are likely to be particularly sensitive to microtubule disruption. Inhibition of microtubule formation or function in the aged neurons by 4HNE might drastically cause microtubule disruption. Previous finding suggests that β-tubulin is a main cellular target protein of 4HNE (Neely et al. 2005). Therefore, we can conclude that ALDH2 protect microtubule or microtubule-related protein that is closely associated with neurite outgrowth through accelerating 4HNE removal. It will be important to assess the microtubule and microtubule-related protein level changes in the future.

Our data suggest not only a protective role of ALDH2 in neurite injury but also a reduction in 4HNE-induced apoptotic neuronal death. Apoptotic cell death has been postulated to be the cause of excessive neuronal cell loss observed in several neurodegenerative diseases. The 4HNE has been shown to induce apoptosis in neurons, including PC12 cells, and as such has been proposed as an important mediator of neuronal death in AD. In our study, treatment with 4HNE increases the expression of markers of neuronal apoptosis can be detected (pyknotic nuclei and the activation of caspase-3). The increasing findings have shown that the activation of caspase-3 has been implicated as a key cell-death protease involved in the execution phase of apoptosis (Budihardjo et al. 1999; Nicholson and Thornberry 1997). However, ALDH2 effectively suppressed 4HNE-induced activation of caspase-3, suggesting that ALDH2 may act upstream of caspase-3 to block apoptosis. It seems that ALDH2 exerts its neuroprotective activities in cultured hippocampal neurons through suppressing them in vitro.

Evidences from previous researches (Poli and Schaur 2000; Nakashima et al. 2003) also indicated that the 4HNE is a key mediator of oxidative stress-induced cell death. 4HNE modulates several mechanisms inducing apoptosis such as, increasing the ROS production and altering mitochondrial respiratory and signal transductions. The excessive ROS will exaggerate oxidative damage, including the mitochondrial malfunction (Sengpiel et al. 1998; Kamat and Devasagayam 2000), which plays a critical role in apoptosis. Oxidative stress caused by 4HNE might be, at least in part, responsible for the alteration of mitochondrial membrane integrity. An increase in ROS production after 4HNE treatment might have contributed to the increased apoptosis in neurons. Previous studies also supported that increased ROS formation and oxidative stress is associated with the collapse of mitochondrial membrane, causing release of cytochrome c and subsequent apoptosis (Musatov et al. 2002; Petrosillo et al. 2001). It is thus reasonable to postulate that the anti-apoptotic effects of ALDH2 may be, at least in part, mediated through decrease of ROS. Several studies have demonstrated that ROS are involved in the apoptotic mechanisms triggered in the process of AD, and may, therefore, contribute to the increased apoptosis found in some models of AD. From our observation, there is a

decrease in the intracellular ROS level, as ROS is thought to be a key mediator in the 4HNE-mediated apoptotic death pathway, suggesting that the ability of ALDH2 to protect against 4HNE-induced neurotoxicity in vitro is enhanced through such a mechanism. Further investigations are required to elucidate these alternative mechanisms.

Blockade of 4HNE by ALDH2 may be a potential therapeutic approach for halting chronic neurodegeneration. It can attenuate the neurotoxicity caused by 4HNE not only at an early stage, but even at late stage. However, the other neuroprotective effects of ALDH2 are presently unknown. Further exploration of ALDH2 therapeutic properties may provide opportunities for novel pharmacological interventions for preventing the consequences of neurodegenerative diseases, such as AD. In addition, the molecular mechanisms that mediate these neuroprotective effects of ALDH2 also remain to be characterized. Interestingly, the anti-apoptotic effect of ALDH2 in various cell lines has been shown to require a combinatorial activation of Akt, and MAPK signal pathways (Xu et al. 2006). It is still unknown whether ALDH2 exerts its antiapoptotic effect in neurons through the same signal pathways. The precious mechanism of ALDH2 neuroprotective effects needs further exploration.

In summary, we have shown that upregulation of ALDH2 expression plays a key role in protecting neurons from neurite damage and apoptosis evoked by 4HNE. Our study provided evidence that overexpression of ALDH2 in neurons significantly lessens 4HNE-induced neurotoxicity, suggesting the therapeutic value of ALDH2 in detoxication of 4HNE, which is essential in the management and prevention of age-related neuronal injury. Future studies will determine the extent of the contribution of ALDH2 to the neuroprotection in vivo. Future study should also focus on accessing the clinical feasibility of ALDH2 in the management of neurodegenerative complications.

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