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Aldehyde dehydrogenase 7A1 (ALDH7A1) attenuates reactive aldehyde and oxidative stress induced cytotoxicity

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ABSTRACT

Mammalian aldehyde dehydrogenase 7A1 (ALDH7A1) is homologous to plant ALDH7B1 which protects against various forms of stress such as increased salinity, dehydration and treatment with oxidants or pesticides. Deleterious mutations in human ALDH7A1 are responsible for pyridoxine-dependent and folinic acid-responsive seizures. In previous studies, we have shown that human ALDH7A1 protects against hyperosmotic stress presumably through the generation of betaine, an important cellular osmolyte, formed from betaine aldehyde. Hyperosmotic stress is coupled to an increase in oxidative stress and lipid peroxidation (LPO). In this study, cell viability assays revealed that stable expression of mitochondrial ALDH7A1 in Chinese hamster ovary (CHO) cells provides significant protection against treatment with the LPO-derived aldehydes hexanal and 4-hydroxy-2-nonenal (4HNE) implicating a protective function for the enzyme during oxidative stress. A significant increase in cell survival was also observed in CHO cells expressing either mitochondrial or cytosolic ALDH7A1 treated with increasing concentrations of hydrogen peroxide (H_2O_2) or 4HNE, providing further evidence for anti-oxidant activity. *In vitro* enzyme activity assays indicate that human ALDH7A1 is sensitive to oxidation and that efficiency can be at least partially restored by incubating recombinant protein with the thiol reducing agent β-mercaptoethanol (BME). We also show that after reactivation with BME, recombinant ALDH7A1 is capable of metabolizing the reactive aldehyde 4HNE. In conclusion, ALDH7A1 mechanistically appears to provide cells protection through multiple pathways including the removal of toxic LPO-derived aldehydes in addition to osmolyte generation.

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1. Introduction

Aldehyde dehydrogenases facilitate the $NAD(P)^+$ dependent oxidation of aldehydes to their corresponding carboxylic acids and NAD(P)H. These enzymes participate in many physiologically important biosynthetic pathways but also play a crucial role in removing toxic aldehydes produced during oxidative stress and the metabolism of xenobiotics. Aldehyde dehydrogenase 7A1 (ALDH7A1), originally named antiquitin, was first identified in plants as an enzyme that was upregulated during various insults such as increased salinity, dehydration and treatment with oxidants or pesticides [1–4]. Deleterious mutations in human ALDH7A1 cause pyridoxine-dependent and folinic acid-responsive seizures [5,6]. In mammals, ALDH7A1 plays a role in lysine catabolism where it metabolizes α-aminoadipic semialdehyde (AASA) to α-aminoadipic acid [7]. Lysine is an essential amino acid and its metabolism is important for the maintenance of cellular nitrogen pools, synthesis of glutamate and formation of ketone bodies [8]. Recently, the enzyme was also found to play a significant role in protecting mammalian cells from hyperosmotic stress, presumably through the generation of the osmolyte betaine from betaine aldehyde [9]. The same study also identified that ALDH7A1 is enzymatically active against a number of lipid peroxidation-derived aldehydes which are formed under oxidative conditions. Hyperosmotic stress is coupled to the generation of reactive oxygen species (ROS) and elevated oxidative stress within the cell [10]. The tight correlation between osmotic and oxidative stress suggests that the cytoprotective functions of ALDH7A1 may be two fold: (1) the generation of osmolytes to counteract osmotic stress and (2) the removal of reactive aldehydes generated as a result of increased oxidative stress.

ROS and lipoxidation products generated during oxidative stress can contribute significantly to cytotoxicity. ROS including hydroxyl

Abbreviations: AASA, α-aminoadipic semialdehyde; 4HNE, 4-hydroxy-2-nonenal; ALDH7A1, aldehyde dehydrogenase 7A1; BME, β-mercaptoethanol; CHO, Chinese hamster ovary; DTT, dithiothreitol; H_2O_2, hydrogen peroxide; LPO, lipid peroxidation; MDA, malondialdehyde; ROS, reactive oxygen species.

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radicals, superoxide and hydrogen peroxide (H_2O_2) are readily formed during oxidative stress and can trigger the oxidative degradation of biological membranes, known as lipid peroxidation (LPO). LPO occurs when free radicals remove electrons from lipids found within cellular membranes, preferentially attacking polyunsaturated fatty acids (PUFAs) [11]. This process produces fatty acid radicals which in turn react with molecular oxygen to form lipid peroxyl radicals. The lipid peroxyl radicals then react with neighboring fatty acids to generate lipid peroxides and additional fatty acid radicals that initiate additional rounds of oxidation. Antioxidants, such as vitamin E, or antioxidant enzymes, including catalase, superoxide dismutase and peroxidases, can terminate redox cycling by directly neutralizing radicals [12]. Aldehydes are produced in large quantities during LPO as a result of lipid chain cleavage and are considered secondary toxic metabolites [13,14]. LPO produces over 200 different aldehydes, many of which are highly reactive and extremely toxic even at low concentrations. Of those produced, propanal, hexanal, MDA and 4HNE are the most abundant [13,14]. Aldehydes can covalently bind to protein and DNA resulting in protein inactivation and DNA damage. Moreover, aldehydes are associated with the pathophysiology of several diseases including Parkinson's disease, Alzheimer's disease, cataract formation, atherosclerosis and alcoholic liver disease, to name a few [15–19]. As such, understanding the enzymes and pathways associated with aldehyde removal has direct relevance to disease prevention and treatment.

The present studies were performed to determine the cytoprotective actions of ALDH7A1 during oxidative stress. More specifically, we examine whether ALDH7A1 can protect mammalian cells from oxidants, such as hydrogen peroxide, and toxic LPO-derived aldehydes.

2. Materials and methods

2.1. Materials

Lipofectamine Plus reagent and hygromycin B were purchased from Invitrogen (Carlsbad, CA). The affinity resin, 5′-AMP-Sepharose 4B, used for fast protein liquid chromotography (FPLC) was obtained from GE Healthcare (Piscataway, NJ). PVDF membranes were purchased from Millipore (Bedford, MA). Complete Protease Inhibitor Cocktail tablets were obtained from Roche (Indianapolis, IN). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Reagents for chemiluminescence were purchased from NEN Life Science Products (Boston, MA). Aldehyde substrates, NAD^+, sodium pyrophosphate, sodium phosphate, potassium phosphate and Amberlyst® 15 ion exchange resin were purchased from Sigma–Aldrich (St. Louis, MO). Allysine ethylene acetal was purchased from Chiralix (Nijmegen, The Netherlands). 4HNE was purchased from Cayman Chemical Company (Ann Arbor, MI). Protein assay reagent was obtained from BioRad (Hercules, CA). All chemicals were of analytical grade. Unless otherwise specified, all tissue-culture media, supplements, assay reagents and buffers were purchased from Invitrogen (Carlsbad, CA) or Fisher Scientific (Hampton, NH).

2.2. Cell culture

Chinese hamster ovary (CHO) cells were grown in F-12 nutrient medium (Ham's) supplemented with 7% (vol/vol) heat-inactivated fetal bovine serum (HI-FBS), 20 mM HEPES (pH 7.4), penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Construction and expression of recombinant plasmids

Mammalian and baculovirus constructs were created using human ALDH7A1 cDNA sequences and either ΔpCEP4Δ or pBlueBac4.5 expression vectors, respectively, as previously described [9]. Both mitochondrial (ΔpCEP4Δ-ALDH7A1v1) and cytosolic (ΔpCEP4Δ-ALDH7A1v2) ALDH7A1 coding sequences were cloned into the ΔpCEP4Δ expression vector. Baculovirus expression constructs were plaque-purified and amplified in Sf9 (*Spodoptera frugiperda*) insect cells by the University of Colorado Cancer Center Protein Production/MoAB/Tissue Culture Shared Core as described previously [20,21].

2.4. Generation of ALDH7A1-transfected cell lines

Stable cell lines were created by transfecting cells with either vector (ΔpCEP4Δ) or mitochondrial ALDH7A1 (ΔpCEP4Δ-ALDH7A1v1) using Lipofectamine Plus reagent as previously described [22]. Stable cell populations were then selected for in media containing hygromycin B (0.4 mg/mL). ALDH7A1 expression was screened by Western blot. Two stable cell lines, CHO-ALDH7A1v1 #14 and #35, were generated and the higher expressing cell line (CHO-ALDH7A1v1 #35) was used in subsequent experiments to determine cytoprotection and LC50 values towards various aldehydes (benzaldehyde, hexanal, octanal, 4HNE, malondialdehyde (MDA) and AASA). Pooled populations of transiently transfected cells were used in SRB assays to compare protective effects of mitochondrial (ALDH7A1v1) and cytosolic (ALDH7A1v2) variants against H_2O_2 and 4HNE. CHO cells were transiently transfected in 100 mm dishes at 80% confluence using Lipofectamine Plus reagent and ΔpCEP4Δ, ΔpCEP4Δ-ALDH7A1v1 or ΔpCEP4Δ-ALDH7A1v2 to create CHO-Vector, CHO-ALDH7A1v1 and CHO-ALDH7A1v2 cells, respectively. Western blotting was then used to determine ALDH7A1 expression levels. These cells were then used to seed multi-well plates for subsequent treatments.

2.5. Western immunoblotting

Cell lysates were prepared using radio-immunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) plus Complete Protease Inhibitor Cocktail. Cells were rocked at 4 °C with RIPA for 15 min then sonicated four times for 10 s. Crude lysates were centrifuged at 15,000 rpm for 10 min to pellet debris and supernatants transferred to a fresh microfuge tube. Lysates (20 μg per well) and recombinant ALDH7A1 (30 ng per well) were separated by SDS–PAGE (12%). Analysis was performed as described previously using polyclonal anti-human ALDH7A1 antibody (1:5000 dilution) and horseradish peroxidase (HRP)-conjugated secondary antibody at 1:5000 [9]. Protein concentrations were determined using BioRad Protein Assay reagent according to the manufacturer's instructions. Densitometry was performed on the resulting Western data using BioRad's Quantity One (version 4.6.5) image analysis software. The results represent the means ± standard deviation from three separate experiments.

2.6. Aldehyde treatments

Unless otherwise stated, aldehyde substrates were obtained from Sigma–Aldrich. AASA was generated from allysine ethylene acetal as described previously [5]. The concentration of AASA synthesized was determined by derivatization to 2-aminobenzaldehyde followed by absorbance measurements at 465 nm [23].

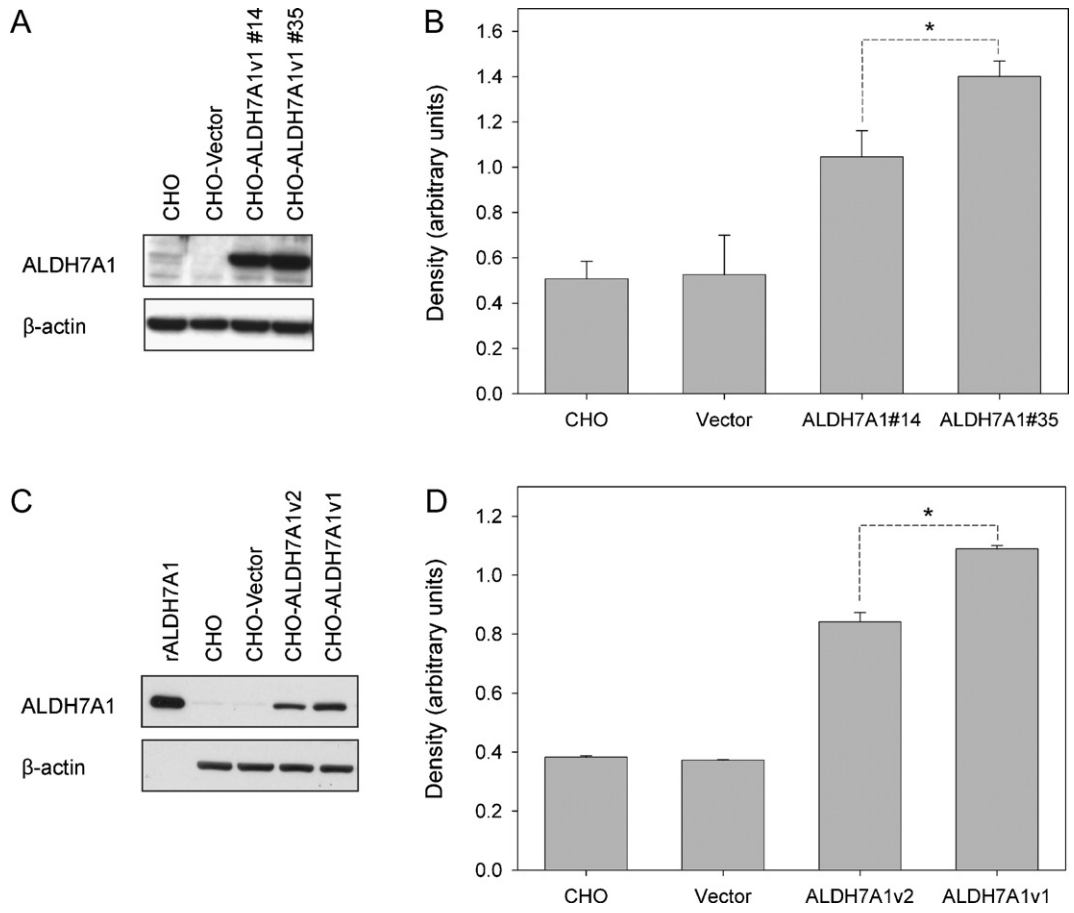

Fig. 1. ALDH7A1 transgene expression in CHO cells. ALDH7A1 protein levels in both stably (A) and transiently (C) transfected cell lysates were analyzed by Western blot analysis. 20 μg cell lysates were ran per well. 30 ng recombinant human ALDH7A1 was included as a positive control (rALDH7A1). Densitometry was then used to compare protein levels between cell lines (B and D). ALDH7A1 protein levels were normalized to the respective β-actin expression. Density is displayed as the means ± standard deviation from three separate experiments (*$p < 0.05$).

The median lethal concentrations (LC50) for various aldehydes were established using CHO-Vector and CHO-ALDH7A1v1 #35 cell lines. Cells were plated in 96-well tissue culture plates and concentration response curves were determined for benzaldehyde (0–20 mM), hexanal (0–20 mM), 4HNE (0–0.32 mM), MDA (0–40 mM) and octanal (0–2 mM) using MTT assays after a 1 h treatment. Data were plotted and LC50 values were calculated for each treatment. Results are presented as a mean ± standard deviation from nine different biological samples from three separate experiments.

For 4HNE and H_2O_2 treatments, transiently transfected CHO-Vector, CHO-ALDH7A1v1 and CHO-ALDH7A1v2 cells were trypsinized and counted with a hemacytometer. They were then used to seed 24-well plates at 2×10^5 cells per well in 1 mL complete growth media and left in the tissue culture incubator overnight. The following day cell confluence was approximately 80%. The media was then carefully removed and replaced with various concentrations of 4HNE (0–100 μM) or H_2O_2 (0–5 mM) in 1 mL phenol red-free DMEM containing 1% fetal bovine serum. Treated cells were incubated for 16 h and images taken using a Nikon Eclipse TS100 inverted microscope equipped with a Nikon DS-Fi1 digital camera (Nikon, Melville, NY). The dishes were then used for subsequent SRB assays. Results represent the combined data from twelve different samples from three separate experiments.

AASA cytotoxicity was determined using stable CHO-Vector and CHO-ALDH7A1v1 #35 cell lines. Cells were treated in 96-well tissue culture plates with increasing concentrations of AASA (0–10 mM) for 24 h and assayed for mitochondrial function by MTT assay. Data are presented as a mean ± standard deviation from 27 different biological samples from three separate experiments. Cell viability is expressed as percent untreated control.

2.7. MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) and SRB (sulforhodamine B) assays

MTT assays were performed as previously described [24]. For SRB assays, 1 mL cold 10% trichloroacetic acid was added to the media after aldehyde treatment. The plates were incubated for 1 h at 4 °C then washed four times with water and allowed to dry overnight at room temperature. The following day 0.5 mL 0.057% (wt/vol) SRB in 1% (vol/vol) acetic acid was added to each well. The plates were left at room temperature for 30 min, rinsed four times with 1% (vol/vol) acetic acid to remove unbound dye then left overnight at room temperature to dry. 1 mL of 10 mM Tris base (pH 10.5) was then added to each well and the plates were shaken for 15 min to dissolve bound dye. Fluorescence measurements were taken at an excitation and emission wavelength of 488 nm and 570 nm, respectively, using a Spectramax Gemini EM platereader (Molecular Devices, Sunnyvale, CA).

2.8. Protein purification

SF9 expressed recombinant human ALDH7A1 was purified via FPLC using an AKITA (GE Health Sciences) equipped with a 5′-AMP-Sepharose 4B affinity column. ALDH purification was performed as previously described by us for other ALDH proteins including

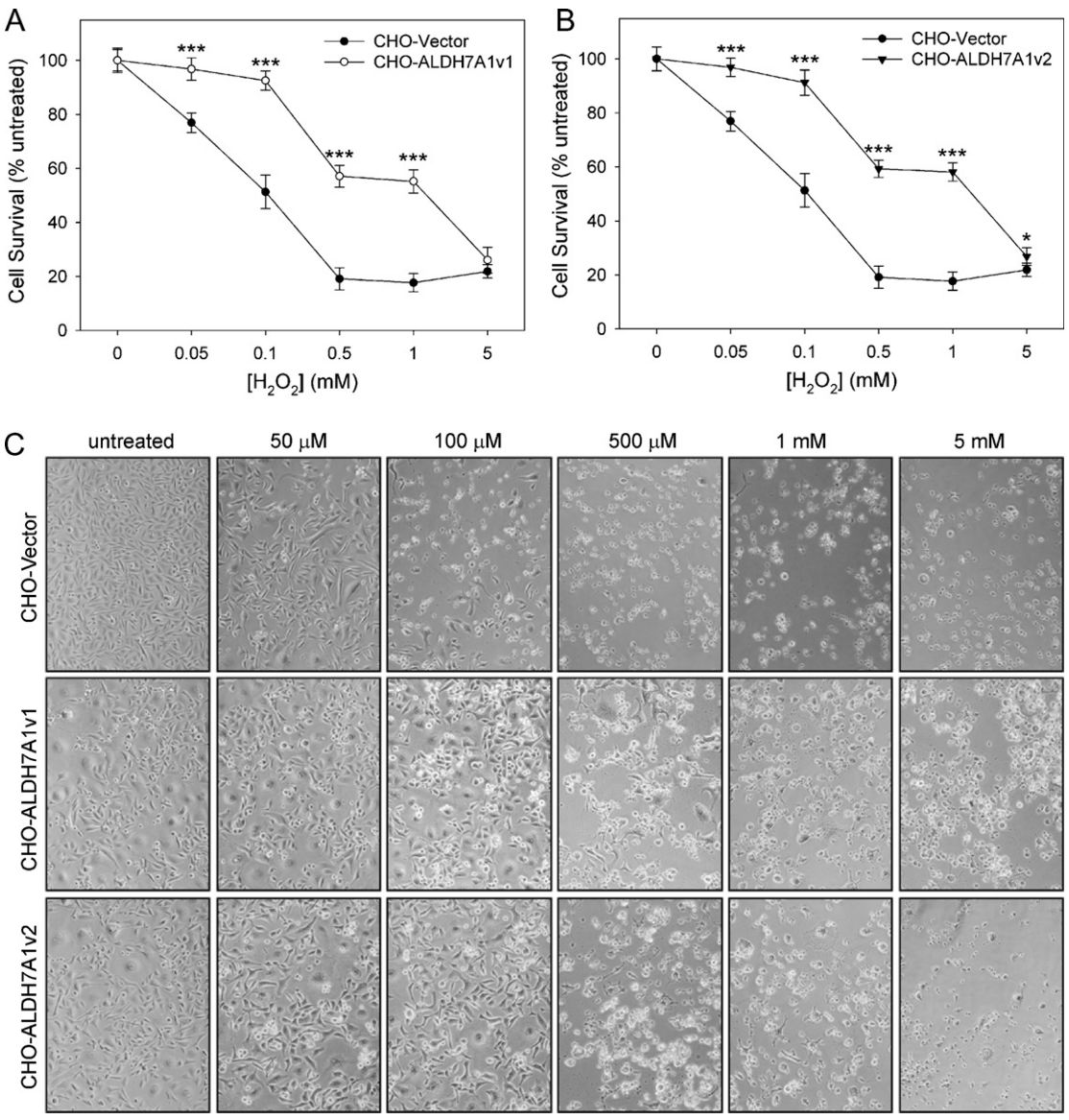

Fig. 2. Mitochondrial and cytosolic ALDH7A1 provide significant protection against hydrogen peroxide (H_2O_2)-induced cytotoxicity. CHO cells were transiently transfected with either mitochondrial (CHO-ALDH7A1v1) (A) or cytosolic (CHO-ALDH7A1v2) (B) ALDH7A1 expression constructs then treated with increasing concentrations of H_2O_2 (0–5 mM) for 16 h and cell survival accessed using SRB assays. Vector transfections were used as a negative control (CHO-Vector). Cell survival data was normalized in respect to untreated control (% untreated). Images of treated cells show significant protection at the indicated concentrations (C). Asterisks immediately above bars denote significant difference from vector control. Data represent means ± SD from triplicate assays ($^*p < 0.05$; $^{***}p < 0.001$).

ALDH3A1 and ALDH1A1 [20,21]. Re-activation of purified recombinant protein was accomplished by incubating the protein in 50 mM BME for 30 min at room temperature prior to kinetic activity measurements.

2.9. ALDH enzymatic activity and kinetic studies

The enzymatic activity of ALDH7A1 was measured spectrophotometrically (Beckman, model DU-640, Fullerton, CA) by monitoring the production of NADH at a wavelength of 340 nm during the oxidation of aldehydic substrates. Activity assays were performed in a 1 mL reaction mixture (25 °C) comprising 100 mM sodium pyrophosphate (pH 8.0), 5 mM BME, 1 mM NAD^+, 1 mM pyrazole and 5 mg recombinant ALDH7A1 protein. The enzymatic reaction was initiated by the addition of aldehydic substrates and measurements were recorded every 5 s for 3 min. For 4HNE kinetic studies, ten substrate concentrations (0–5 mM) were assayed with at least four concentrations both above and below the apparent K_m. The resulting rates were fitted to the Michaelis–Menten equation using SigmaPlot (version 7.0) software.

2.10. Data analysis

Data were expressed as mean ± standard deviation (SD). Different data group comparisons were performed by Student's unpaired *t*-test. All data analyses including enzyme kinetics were carried out using SigmaPlot (version 7.0) software. Observed differences were regarded as significant at $p < 0.05$.

3. Results

3.1. ALDH7A1 expression in CHO cells

Stable and transient expression of ALDH7A1 in CHO cells was verified by Western blot (Fig. 1A and C). Expression levels in both stable and transiently transfected cell lines were compared using

Table 1
LC50 values for toxic aldehydes in CHO-Vector and CHO-ALDH7A1v1 cell lines. Control (CHO-Vector) and mitochondrial ALDH7A1 (CHO-ALDH7A1v1 #35) cell lines were plated in 96-well tissue culture plates and treated with benzaldehyde (0–20 mM), hexanal (0–20 mM), 4HNE (0–0.32 mM), MDA (0–40 mM) and octanal (0–2 mM). MTT assays were performed after 1 h exposure. Median lethal concentration (LC50) values were determined using SigmaPlot software. Data are presented as a mean ± standard deviation from nine different biological samples from three separate experiments (***$p < 0.001$).

Treatment	CHO-Vector	CHO-ALDH7A1v1
Benzaldehyde	14.4 ± 0.44	15.1 ± 0.88
Hexanal	2.8 ± 0.08	7.1 ± 0.40***
Octanal	1.8 ± 0.39	1.1 ± 0.01
4-HNE	0.081 ± 0.003	0.125 ± 0.005***
MDA	37.3 ± 0.40	36.2 ± 0.43

*** $p < 0.001$.

densitometry after being normalized to the respective β-actin bands (Fig. 1B and D). Results obtained indicated that the total ALDH7A1 protein levels were approximately 35% higher in CHO-ALDH7A1v1 #35 when compared to CHO-ALDH7A1v1 #14 cells. The higher expressing cell line (CHO-ALDH7A1v1 #35) was used in subsequent treatment experiments. In transiently transfected cells, cellular ALDH7A1 protein levels were approximately 20% higher in the CHO-ALDH7A1v1 (mitochondrial) cells than the ALDH7A1v2 (cytosolic) cell line. Mitochondrial and cytosolic subcellular localization for the ALDH7A1 variants cloned into the given expression vectors were characterized previously [9,25].

3.2. ALDH7A1 mediated cytoprotection against reactive aldehydes

CHO-Vector and CHO-ALDH7A1v1 #35 cells were treated with increasing concentrations of benzaldehyde (0–20 mM), hexanal (0–20 mM), 4HNE (0–0.32 mM), MDA (0–40 mM) and octanal (0–2 mM). Following aldehyde treatment, MTT assays were performed to determine the ability of ALDH7A1 expression to protect cells from cytotoxicity (Table 1). The calculated LC50 values for CHO-Vector treatments were 14.4 mM, 2.8 mM, 1.8 mM, 81 μM and 37.3 mM for benzaldehyde, hexanal, octanal, 4HNE and MDA, respectively. CHO-ALDH7A1v1 #35 cell treatments gave LC50 values of 15.1 mM, 7.4 mM, 1.1 mM, 125 μM, 36.2 mM for benzaldehyde, hexanal, octanal, 4HNE and MDA, respectively. ALDH7A1 expression provided significant protection ($p < 0.001$) against both hexanal and 4HNE. Significant protection was not observed in benzaldehyde, octanal or MDA treated cells.

3.3. Mitochondrial and cytosolic ALDH7A1 expression in CHO cells treated with H_2O_2 or 4HNE

CHO cells transiently transfected with either the mitochondrial (CHO-ALDH7A1v1) or cytosolic (CHO-ALDH7A1v2) forms of human ALDH7A1 were treated with increasing concentrations of either H_2O_2 (0–5 mM) (Fig. 2) or 4HNE (0–100 μM) (Fig. 3). SRB assays were then used to analyze cell survival. CHO-Vector cells exhibited comparable sensitivity to H_2O_2 as a number of cell lines identified within the literature [26–31]. Significant protection ($p < 0.001$) was observed at 50 μM, 100 μM, 500 μM and 1 mM H_2O_2 in cells expressing either the mitochondrial or cytosolic forms of the protein (Fig. 2A and B). Significant protection ($p < 0.05$) was also observed at 5 mM in cell line expressing the cytosolic variant (Fig. 2B). The degree of protection was also evident in images taken of the treated cells at the concentrations mentioned above (Fig. 2C).

Both cell lines were additionally treated with increasing concentrations of the reactive aldehyde 4HNE. Significant protection was observed in cells expressing mitochondrial ALDH7A1 at 25 μM ($p < 0.001$), 50 μM ($p < 0.001$) and 100 μM ($p < 0.01$) 4HNE (Fig. 3A). The cytosolic variant also provided significant protection against 4HNE treatment at 25 μM ($p < 0.01$), 50 μM ($p < 0.001$) and 100 μM ($p < 0.01$) (Fig. 3B). Images of treated cells further support significant protection at these concentrations (Fig. 3C).

The degree of protection conferred for mitochondrial and cytosolic ALDH7A1 was also compared for each treatment. No significant differences were noted between the H_2O_2 treated cells at any concentrations. The mitochondrial variant provided significantly more protection than cytosolic ALDH7A1 in cells treated with 25 μM ($p < 0.001$), 50 μM ($p < 0.01$) and 100 μM ($p < 0.01$) 4HNE. However, transgene expression levels differ slightly between the two cell populations making direct comparisons difficult.

3.4. ALDH7A1 cytoprotection against α-aminoadipic semialdehyde (AASA) treatment

CHO cells stably over expressing mitochondrial ALDH7A1 (CHO-ALDH7A1v1 #35) were treated with increasing concentrations of the lysine degradation product AASA (0–10 mM) (Fig. 4). MTT assays were then performed to determine aldehyde toxicity and the ability of ALDH7A1 to attenuate these effects. It was difficult to determine an accurate LC50 value due to the limitations in the maximum treatment concentrations of AASA after synthesis. However, we were able to identify significant protection ($p < 0.05$) at the two highest concentrations of AASA (5 and 10 mM).

3.5. Recombinant enzyme activity against 4-hydroxy-2-nonenal (4HNE) and effect of activation with β-mercaptoethanol (BME)

Previous studies have shown that many enzymes, including mitochondrial ALDHs, are susceptible to oxidative inactivation. As such, incubation with reducing agents such as dithiothreitol (DTT) and BME can restore catalytic activity [22,32–35]. The effect of BME incubation on ALDH7A1 activity was analyzed using propanal as a substrate. In previous studies ALDH7A1 exhibited a K_m and V_{max} of 647.4 μM and 69.9 nmol NADH/min/mg, respectively, towards this substrate [36]. Incubating recombinant ALDH7A1 with BME prior to measuring enzymatic activity resulted in an approximately four fold increase in the initial reaction velocity (V_0) at all propanal concentrations used (0.25, 0.75 and 1.0 mM) when compared to non-activated samples (Fig. 5). Non-activated, recombinant human ALDH7A1 did not exhibit activity towards 4HNE. However, activation of recombinant enzyme with BME restored activity towards the substrate. A K_m of 119.5 ± 16.8 μM, V_{max} of 323.5 ± 13.4 nM/min/mg and K_m/V_{max} of 2.71 were determined for 4HNE by monitoring the production of NADH at a wavelength of 340 nm.

4. Discussion

Mitochondrial ALDH7A1 was previously shown to protect mammalian cells against hyperosmotic stress [9]. Herein, we report additional cytoprotective functions for ALDH7A1 against oxidative stress and LPO-derived aldehydes. Reactive oxygen species (ROS) production is an unavoidable consequence of aerobic metabolism. Hydrogen peroxide (H_2O_2) is an uncharged, relatively stable and freely diffusible ROS. The compound is generated physiologically during mitochondrial respiration predominantly by superoxide dismutase (SOD) [37]. Many other enzymes, including monoamine oxidases (MAOs) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, also contribute to H_2O_2 production. Elevated H_2O_2 within cells and tissues can have a variety of pathological consequences. H_2O_2 can be converted to highly reactive hydroxyl radicals ($OH^•$) which can then attack DNA, proteins and lipids resulting in enzyme inactivation, DNA strand breakage and

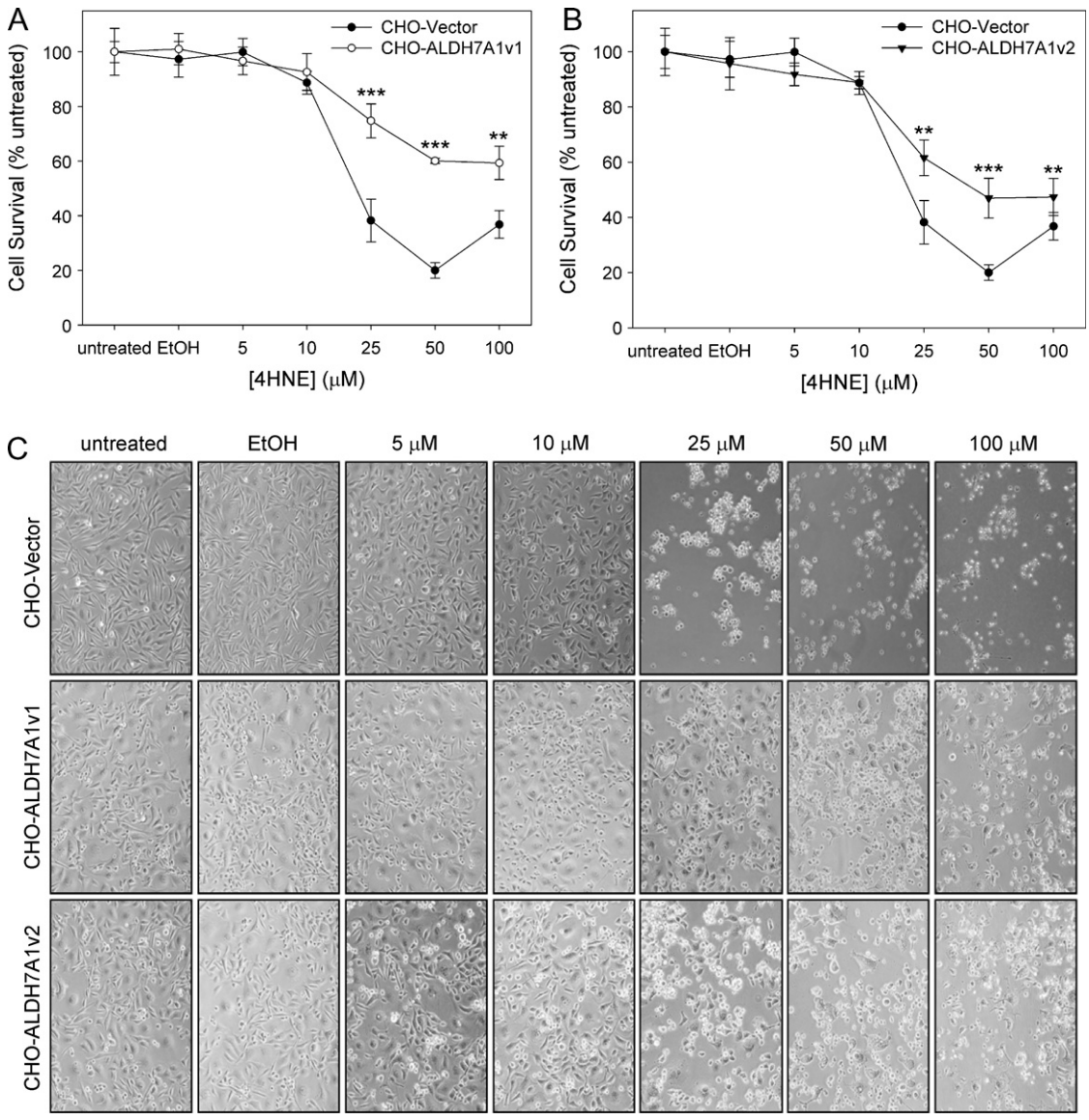

Fig. 3. Mitochondrial and cytosolic ALDH7A1 provide significant protection against 4-hydroxy-2-nonenal (4HNE)-induced cytotoxicity. CHO cells were transiently transfected with either mitochondrial (CHO-ALDH7A1v1) (A) or cytosolic (CHO-ALDH7A1v2) (B) ALDH7A1 expression constructs then treated with increasing concentrations of 4HNE (0–100 μM) for 16 h and cell survival accessed using SRB assays vector transfections were used as a negative control (CHO-Vector). EtOH alone was used to determine the effect of this vehicle. Cell survival data was normalized in respect to untreated control (% untreated). Images of treated cells support significant protection at the indicated concentrations (C). Asterisks immediately above bars denote significant difference from vector control. Data represent means ± SD from triplicate assays (**$p < 0.01$; ***$p < 0.001$).

lipid peroxidation, respectively. Lipid peroxidation (LPO) describes the oxidative degradation of biological membranes. LPO is self-perpetuating process and produces over 200 types of aldehydes [38]. Many LPO-derived aldehydes can covalently bind, or adduct, biological macromolecules including DNA, proteins and lipids [13]. Similar to the damage caused by hydroxyl radicals, LPO aldehyde adduction can inactivate enzymes and damage chromosomal DNA. Furthermore, aldehydes are potent electrophiles that readily bind glutathione (GSH). As oxidative stress persists, cellular GSH pools are depleted and intracellular redox balance becomes impaired. Unhindered, the combined effects of hydroxyl radicals and LPO-derived aldehydes can significantly upset cellular homeostasis leading to cell death and tissue damage. The ALDH7A1-mediated removal of LPO-derived aldehydes could therefore represent an additional cytoprotective mechanism by which the cell preserves glutathione pools counteracting the damaging effects of oxidative stress. Overexpression of mitochondrial ALDH7A1 in CHO cells (CHO-ALDH7A1v1) provided protection against the reactive aldehydes hexanal and 4HNE. CHO-Vector and CHO-ALDH7A1v1 cell lines were treated with increasing concentrations of benzaldehyde, hexanal, octanal, 4HNE and MDA. Following treatment, MTT assays were used to calculate LC50 values for each treatment in the two cell lines. Hexanal treated CHO-Vector and CHO-ALDH7A1v1 cell lines exhibited LC50 values of 2.8 mM and 7.1 mM, respectively. Similar results were observed in cells treated with 4HNE. Calculated LC50 values for CHO-Vector and CHO-ALDH7A1v1 cell lines were 81 μM and 125 μM 4HNE, respectively. These data were somewhat surprising seeing how previous studies did not identify activity towards this substrate [39]. Protection was not found in cells treated with the aromatic aldehyde benzaldehyde or the three carbon dialdehyde MDA, which was not unexpected seeing how the enzyme was previously shown to have either very low affinity or no activity towards these substrates [9,39]. Surprisingly, we also did not see protection in octanal treated cells. Octanal is a high affinity

substrate for ALDH7A1 and previous studies revealed that the K_m for octanal is significantly lower than the K_m for hexanal, 17.5 μM vs. 39.1 μM, respectively [9]. Despite a lower K_m, the calculated V_{max} for octanal was over three fold less than hexanal resulting in a overall lower catalytic efficiency towards the substrate when compared to hexanal, which may explain the observed difference in protection [9].

A mitochondrial (ALDH7A1v1) and cytosolic (ALDH7A1v2) form of human ALDH7A1 have been identified [9,25]. Furthermore, distinct localization patterns suggest that the enzyme may be associated with different pathways and, therefore, substrates within specific cell-types and tissues. To shed light on whether subcellular localization influences protection, CHO cells were transfected with either mitochondrial or cytosolic ALDH7A1 then treated with increasing concentrations of H_2O_2 and 4HNE. Following treatment, SRB assays were performed to determine cell survival. Results from these experiments revealed that both the mitochondrial and cytosolic variants confer significant protection against these toxic compounds. There was no significant difference in the degree of protection noted between the mitochondrial and cytosolic protein expressing cell lines treated with H_2O_2. Interestingly, a significant difference in overall protection was observed between the two ALDH7A1 expressing cell lines treated with 4HNE. However, subsequent densitometry analyses indicated higher transgene expression in the mitochondrial cell line, which could account for the observed experimental differences. Nevertheless, it is interesting to postulate that subcellular localization could have differential effects on cytoprotection depending on insult type.

The linear aliphatic aldehyde hexanal and the α, β-unsaturated aldehyde 4HNE are two of the predominant aldehydes generated during LPO [13,14]. These studies reveal that ALDH7A1 provides significant protection against both compounds. Under normal physiological conditions, the basal levels of 4HNE are very low (<1 μM) [13]. However, 4HNE concentrations vary greatly, especially during oxidative stress where concentrations can increase significantly. Hexanal does not exhibit the same toxicity as 4HNE but concentrations were shown to be approximately nine fold higher than 4HNE under various types of stress [14]. Moreover, 4HNE and other lipoxidation product levels increase with proximity to lipid membranes, which are the major site of synthesis. The concentration of 4HNE within the lipid membranes of peroxidised microsomes were calculated to be approximately 4.5 mM [40]. Furthermore, the average half-life of 4HNE is approximately 2 min, as such, it is capable of diffusing significant distances, in effect, establishing a concentration gradient from the site of production [13]. Previous studies looking at the seabream fish ALHD7A1 did not detect activity towards 4HNE [39]. However, the range of 4HNE concentrations used in the assay was 1–32 mM. At these concentrations there is a high likelihood that 4HNE would completely inactivate the enzyme. The calculated K_m of human ALDH7A1 towards 4HNE was 119.5 μM. This value does not strongly support a catalytic function under physiological conditions. Moreover, related ALDH superfamily members metabolize 4HNE with much higher affinity, including mitochondrial ALDH2 which exhibits K_m and V_{max} values of 14.3 μM and 3.5 nmol/min/mg, respectively [41,42]. Interestingly, many LPO-derived aldehydes such as 4HNE, acrolein, crotonaldehyde and MDA are known to inhibit aldehyde dehydrogenase activity [43–46]. 4HNE has been shown to reversibly inhibit ALDH2 and at high concentrations (>50 μM) the enzyme is completely inactivated [47]. It is therefore reasonable to postulate that as 4HNE concentrations increase to levels that inhibit low K_m enzymes, such as ALDH2, ALDH7A1 and other high K_m ALDH enzymes might then play an important role in metabolizing this cytotoxic compound. To date, ALDH7A1 is unique among ALDH enzymes in that it is expressed at high levels in multiple subcellular compartments including the mitochondria, cytosol and nucleus

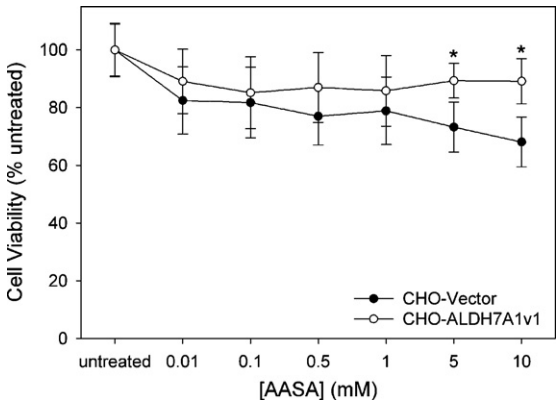

Fig. 4. Mitochondrial ALDH7A1 expression provides significant protection against α-aminoadipic semialdehyde (AASA)-induced cytotoxicity. CHO cells stably transfected with either vector (CHO-Vector) or mitochondrial ALDH7A1 (CHO-ALDH7A1v1 #35) were treated in 96-well tissue culture plates with various concentrations of AASA (0–10 mM) for 24 h and assayed for mitochondrial function by MTT assay. Cell viability data was normalized in respect to untreated control (% untreated). Asterisks immediately above bars denote significant difference from vector control. Data are presented as a mean ± standard deviation from 27 different biological samples from three separate experiments (*$p < 0.05$).

[9,25]. Given the high concentrations and widespread distribution of 4HNE, the extensive subcellular localization of ALDH7A1 further supports a potentially important pathophysiological role for 4HNE metabolism in response to pro-oxidant exposure.

The lysine catabolite, AASA, is thought to be a major physiological substrate for mammalian ALDH7A1. To date, very little is known about the inherent toxicity associated with this aldehyde. Our data indicates that AASA was only mildly toxic at relatively high concentrations. The physiological concentration of this aldehyde within tissues in not known. However, the levels in human plasma are reported to be less than 0.2 μM [48]. In patients with null mutations in ALDH7A1 reported plasma AASA concentrations are between 0.5 and 14 μM [48]. At these levels it does not appear the aldehyde itself exhibit toxicity. However, in pyridoxine-dependent epilepsy (PDE) the pathophysiology of increased AASA concentrations is believed to manifest via the conjugation of AASA to pyridoxal phosphate (PLP), or vitamin B6, resulting in severe vitamin B6 deficiency. In tissues the levels of free lysine are reported to be approximately 1000 μM, 650 μM and 200 μM in muscle, liver and plasma, respectively [49–51]. This suggests that even under conditions highly

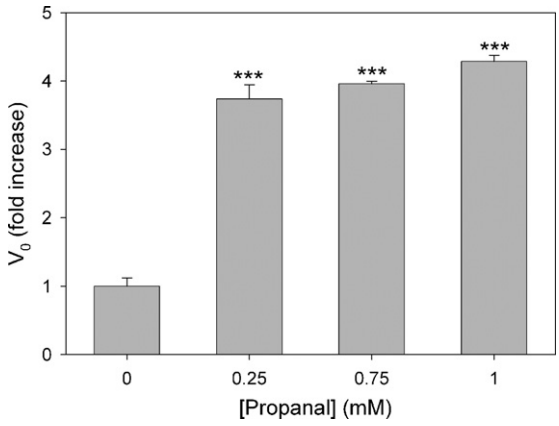

Fig. 5. ALDH7A1 activity is sensitive to oxidation. Incubation of ALDH7A1 with the reducing agent BME restores catalytic activity. *In vitro* activity assays with recombinant human protein revealed an approximately 4 fold increase in initial velocity (V_0) at all propanal concentrations (0.25 mM, 0.75 mM & 1.0 mM) tested when compared to non-activated enzyme. Asterisks immediately above bars denote significant difference from vector control (***$p < 0.001$).

favoring lysine oxidation to AASA the concentrations would probably not raise to toxic levels (i.e. >5 mM).

Intracellular redox state and oxidative stress are known to significantly affect enzyme activity [52]. Loss of enzyme activity as a result of protein oxidation in many cases can be reversed with a thiol reducing agent, such as BME, which restores sulfhydryl groups [32–34]. The substantial increase in ALDH7A1 catalytic activity after incubation with BME suggests the enzyme is sensitive to oxidation. Moreover, recombinant ALDH7A1 was unable to metabolize 4HNE without prior activation with the reducing agent. Oxidants such as H_2O_2 are known to significantly inhibit erythrocyte aldehyde dehydrogenase I activity [53]. This implies that as oxidative stress increases within the cell the activity and substrate specificity of ALDH7A1 may be significantly altered. In previous studies, it was hypothesized that human ALDH7A1 plays an important protective role during hyperosmotic stress presumably through the generation of the cellular osmolyte betaine from betaine aldehyde [9]. The study also identified that ALDH7A1 is capable of metabolizing a number of known LPO-derived aldehydes, suggesting a role for ALDH7A1 during cellular defense against oxidative stress. The current study expands upon these findings and confirms that ALDH7A1 enzyme activity is cytoprotective under oxidative conditions where LPO and subsequent aldehyde levels are elevated. Mechanistically, the removal of LPO-derived aldehydes by ALDH7A1 could have multiple cytoprotective functions. By lowering aldehyde concentrations within the cell, ALDH7A1 metabolic activity should reduce the need for GSH conjugation and help maintain intracellular GSH levels. Moreover, the ALDH7A1 mediated removal of reactive aldehydes, such as hexanal and 4-HNE, may lower the extent of macromolecule adduction thereby lessening pathogenic consequences associated with enzyme inactivation. In conclusion, data obtained from these studies supports an important role for ALDH7A1 in protecting cells from the damaging effects of oxidative stress and future studies are needed to elucidate the exact mechanisms underlying these observations.

Conflict of interest

The authors declare no conflict of interest associated with this manuscript.

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