Role of Aldehyde Dehydrogenase Isozymes in the Defense of Rat Lens and Human Lens Epithelial Cells against Oxidative Stress

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Purpose. 4-Hydroxynonenal (HNE), a metastable lipid peroxidation product, is highly toxic to various cell types if not detoxified. Because of its constant exposure to light, the ocular lens continuously generates reactive oxygen species which, under conditions of oxidative stress, may lead to excessive lipid peroxidation and consequent formation of lipid-derived aldehydes (LDAs) such as HNE. The contribution of various isozymes of aldehyde dehydrogenase (ALDH) to the oxidation of LDAs has never been systematically investigated in the lens. The present study was undertaken to ascertain the role of ALDH1A1 and -3A1 in HNE metabolism and HNE-induced toxicity in cultured human lens epithelial cells (HLECs) and in rat and mouse lenses.

Methods. The metabolism of ^{3}H-HNE was studied in ALDH3A1-knockout mouse lens and in HLECs transfected with ALDH1A1- or -3A1-specific antisense RNA and short interfering (Si)RNA. Appropriate controls were used, including wild-type mouse lens, scrambled oligonucleotides, and a transfection reagent. Transfected HLECs were exposed to oxidative stress (Fenton reaction) or HNE (30 μM) for 3 hours. Toxicity parameters, such as cell viability, apoptosis, and protein-HNE adducts and oxidation of exogenously added ^{3}H-HNE were measured. Rat lenses were transfected with the SiRNA specific to ALDH1A1, and oxidation of ^{3}H-HNE and the susceptibility of the transfected lenses to oxidation-induced opacification were measured.

Results. Rat lenses transfected with ALDH1A1-specific SiRNA, or cultured in the presence of the ALDH inhibitor cyanamide/disulfiram and subjected to oxidative stress displayed accelerated loss of transparency and a diminished capacity to oxidize HNE. Similarly, inhibition of ALDH1A1 in HLECs by ALDH1A1-specific antisense RNA or SiRNA was associated with decreased oxidation of ^{3}H-HNE and increased susceptibility of the cells to oxidative damage, including apoptosis. Furthermore, ^{3}H-HNE metabolism and HNE-induced toxicity were not affected in ALDH3A1-specific SiRNA- or antisense RNA-treated rat lenses, HLECs, or ALDH3A1-null mouse lenses.

Conclusions. The results suggest that, under oxidative stress, HNE produced in the lens epithelium can cause toxicity and thus contribute to oxidation-induced cataractogenesis. Furthermore, the studies indicate that ALDH1A1 is a critical isozyme for maintaining clarity in human, rat, and mouse lenses. (*Invest Ophthalmol Vis Sci.* 2005;46:259–267) DOI:10.1167/iovs.04-0120

Although it has been clearly established that oxidative stress plays a critical role in the pathogenesis of cataracts of various etiologies, including those due to aging, UV light, x-rays, selenite, diquat, and diabetes (for reviews, see Refs. 1,2), the mechanism(s) through which reactive oxygen species (ROS)–induced injury is propagated remains poorly understood. Because ROS have extremely short half-lives, they cause damage, principally at their site of formation. Of the various targets of ROS, membrane lipids appear to be the most vulnerable. Nevertheless, the mechanisms by which lipid peroxidation contribute to lens opacification remain unclear. Lipid peroxidation generates high levels of alkoxyl radicals that degenerate into saturated and unsaturated lipid-derived aldehydes (LDAs). There is increasing evidence suggesting that LDAs formed in nonlenticular tissues by lipid peroxidative reactions mediate the biological effects of free radicals (for reviews, see Refs. 3,4). By acting as toxic messengers, LDAs propagate oxidative stress and may be responsible for a significant portion of the tissue damage ascribed to their radical precursors. We and other investigators have demonstrated that increased LDAs are formed when oxidants overwhelm the antioxidative capacity of various tissues, including the ocular lens.[5–7]

The hydroxyalkenals such as 4-hydroxynonenal (HNE) have three main functional groups—the α,β-unsaturated carbon-carbon double bond, the aldehyde group, and the hydroxyl group—that provide unusually high reactivity to these metabolites. Protein-HNE adducts have been used as markers of oxidative stress. Several studies have demonstrated the involvement of protein-HNE adducts in the pathogenesis of various diseases initiated by oxidative stress.[8–10] We have shown earlier that HNE is cataractogenic[11] at micromolar concentrations, and can induce apoptosis in HLECs.[12] It follows that to prevent oxidative stress-induced toxicity, LDAs such as HNE must be detoxified. The detoxification routes have been studied both in nonocular[13–16] and ocular tissues, such as lens and HLECs.[17] We have shown that the lens can efficiently detoxify HNE under physiological conditions. However, oxidative stress may impair HNE detoxification, resulting in increased protein-HNE formation and toxicity. While studying the metabolism of HNE in rat lens and HLECs, we observed that blocking the oxidative pathway with inhibitors of aldehyde dehydrogenase (ALDHs) did not result in a compensatory increase in the other HNE-detoxification pathways.[17] This suggests that the oxidative pathway of HNE metabolism catalyzed by ALDH could be

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crucial in maintaining lens transparency during oxidative stress.

ALDHs are the products of a large gene family and catalyze irreversible oxidation of a variety of biological aldehydes, including products of lipid peroxidation (for reviews, see Refs. 18–20). The ALDH isozymes are differentially expressed in various tissues, with ALDH1A1 and -3A1 considered the isozymes necessary for detoxification of HNE in the eye. In human lens, expression of ALDH1A1 is higher than that of ALDH3A1.[21–23] The major cytotoxic lipid-aldehyde, HNE, is generated in abundance in oxidatively stressed HLECs.[7] The lens epithelium plays a critical role in maintaining lens transparency. In the current study, we confirmed the importance of the oxidative route in detoxifying HNE in lens and HLECs and extended our results to implicate ALDH1A1 as the critical ALDH isozyme.

METHODS

Cell culture medium, fetal bovine serum, antibiotics, and all chemicals not otherwise specified were purchased from Sigma-Aldrich (St. Louis, MO). HNE and protein-HNE antibodies were obtained from Cayman Chemicals (Ann Arbor, MI). A cell viability ELISA kit (Cell Death ELISA) was purchased from Roche Diagnostics (Mannheim, Germany). ALDH1- and ALDH3-specific short interfering (Si)RNAs were designed and synthesized by Qiagen-Xeragon (Germantown, MD) and Dharmacon Research (Lafayette, CO), respectively. Antisense RNAs specific to various ALDH isozymes were synthesized by Molecular Research Laboratory (Herndon, VA). ALDH3A1-knockout mice and wild-type mice were generated as described previously by us.[24] Antibodies against human liver ALDH1A1, raised in rabbits, were generously provided by Henry Weiner (Purdue University, West Lafayette, IN).

Lens Culture

The NIH guidelines and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were strictly adhered to for the welfare of the animals. Rat or mouse lenses were dissected and washed twice with sterile PBS and then cultured in 1.0 mL medium 199 (Invitrogen-Gibco, Grand Island, NY) containing 100 U penicillin and 100 μg/mL streptomycin under sterile conditions in an atmosphere of 5% CO_2 and 95% humidity at 37°C. Lenses from wild-type and ALDH3A1-knockout mice and rats were divided into various groups with two mouse lenses or one rat lens in each and were exposed to either 100 μM HNE or the Fenton reaction (100 μM ferrous ammonium sulfate, 100 μM H_2O_2, 2 mM adenosine diphosphate [ADP]) for 48 hours or were used to study the metabolism of HNE. In the experiments with cyanamide/disulfiram, rat lens was incubated for 1 hour with 1 mM cyanamide or 50 μM disulfiram to inhibit ALDH. Cyanamide and the more potent inhibitor, disulfiram, inhibit various ALDH isozymes to different extent and are regarded as general inhibitors of these enzymes.[25,26]

Cell Culture

Human lens epithelial cells (HLECs) with extended lifespan were generously provided by Usha Andley (Washington University, St. Louis, MO) and were used in this study, as we previously described.[12,17] Briefly, cells were grown in Eagle's MEM (Sigma-Aldrich) containing 50 μg/mL gentamicin and 20% fetal bovine serum in an atmosphere of 5% CO_2 in air at 37°C. Cell survival was determined by trypan blue staining. Counting was performed with a hemocytometer. Cell viability is represented as the percentage of the number of cells alive/number of total cells. The cells transfected with the antisense or SiRNA specific to ALDHA1 and -3A1 were exposed to either 50 μM HNE for 3 hours or the Fenton reagent for 16 hours or were used to study the metabolism of HNE.

Transfection of HLECs with SiRNA or Antisense RNA Specific to ALDH1A1 or -3A1

To understand the contribution of ALDH1A1 and -3A1 to HNE-induced toxicity and its metabolism, we selective silenced these enzymes using antisense RNA or SiRNA against the isozymes. The specificity of the antisense RNA or SiRNA was assessed by transfecting the cells with the corresponding scrambled oligo or nonsilencing SiRNA. The human ALDH3A1-specific SiRNA duplex, r(GAA GAG CUU CGA GAC UUU C) d(TT): r(G AAA GUC UCG AAG CUC UUC) d(TT), (target sequence; 5′-AAG AAG AGC UUC GAG ACU UUC-3′) was synthesized by Dharmacon Research. The antisense RNA specific to human ALDH3A1 (5′-CCC GTG CTG CCC GTG TAC AGG-3′) and its corresponding scrambled oligos (5′-GGA CAT GTG CCC GTC GTG CCC-3′) were synthesized by Molecular Research Laboratory. Similarly, antisense RNA specific to human ALDH1A1, (5′-GCC TGA GGA TGA CAT TTC TG-3′) and its corresponding scrambled oligo, 5′-GTC TTT ACA GTA GGA GTC CG-3′, were designed and synthesized by Molecular Research Laboratory. Human ALDH1A1-specific SiRNA duplex, r(CUG GGA GAG UAC GGU UUC C)d(TT): r(G GAA ACC GUA CUC UCC CAG)d(TT), (target sequence; 5′-AAC TGG GAG AGT ACG GTT TCC-3′) was synthesized by Qiagen-Xeragon. The nonsilencing SiRNA duplex, r(UUC UCC GAA CGU GUC ACG U) d(TT): r(A AGA GGC UUG CAC AGU GCA) d(TT), (target sequence; 5′-AAT TCT CCG AAC GTG TCA CGT-3′) was also synthesized by Qiagen-Xeragon. The concentrations of oligos or SiRNA and time of incubations were optimized before conducting the final experiments. The day before the transfection, 0.6×10^6 HLECs were seeded overnight in regular media without antibiotics. Cells were transfected with 100 nM oligonucleotides or SiRNA using oligofectamine reagent obtained from Invitrogen Life Technology (Carlsbad, CA) for 4 hours or using the a kit (Targefect SiRNA transfection kit) obtained from Targeting Systems (Santee, CA) for 2 hours, respectively. To check the transfection efficiency, 0.6×10^6 cells were seeded overnight and transfected for 2 hours with 100 nM Cy3 double-stranded (ds)RNA (fluorescence-labeled), by using the SiRNA kit (Targefect; Targeting Systems). After the transfected cells were washed, the transfection efficiency was evaluated by visualization of the red fluorescence of Cy3 in the cells. After transfection of the cells with oligos or SiRNA, the transfection medium was replaced with regular medium containing 10% serum and antibiotics. Cells were harvested 72 hours after transfection—a time predetermined to achieve maximum ALDH silencing. The silencing effect of the oligos or SiRNA on the specific enzymes was determined by RT-PCR. Total RNA was isolated (Total RNA Isolation Kit; Qiagen, Valencia, CA). cDNA was synthesized with oligodT primers obtained from Ambion (Austin, TX), total RNA (12.5 μg), and RNase reverse transcriptase (Superscript II) obtained from Invitrogen-Life Technology (Gaithersburg, MD), according to the manufacturer's protocol.

Reverse Transcription–Polymerase Chain Reaction

The following primers were used to synthesized the PCR products corresponding to human ALDH3A1: upstream primer, AGA GTT CTA CGG GGA AGA TGC TAA G; downstream primer, GCA AGG TGA TGT GGA GGA TGA C. Primers specific for human ALDH1A1 were designed as follows: 5′ primer, TCA CAG GAT CAA CAG AG (nucleotide [nt] 731-747); 3′ primer, GTA GAA TAC CCA TGG TGT GC (nt 892-872); the expected size of the PCR product is 161 bp. PCR was performed with 2 μL (of 50 μL cDNA synthesized) and *Taq* polymerase from Roche Applied Science (Indianapolis, IN). GAPDH was used as an internal standard. The following primers were used for GAPDH: 5′primer, TGA AGG TCG GAG TCA ACG GAT TTG GT; 3′primer, CAT GTG GGC CAT GCA GGT CCA CCA C. As described later in the article, cells were assessed for (1) metabolism of ^{3}H-HNE, to evaluate a decrease in oxidation and (2) toxicity (cell viability and apoptosis), after they were exposed to 40 μM HNE or 100 μM H_2O_2 for 4 hours.

Transfection of Rat Lenses with SiRNA

The culture lenses were transfected with 100 nM of Cy3 dsRNA, rat ALDH1A1-specific SiRNA, r(GAG UGU UGA GCG AGC CAA G)d(TT): r(C UUG GCU CGC UCA ACA CUC)d(TT), (target sequence; AAG AGT GTT GAG CGA GCC AAG), or nonsilencing SiRNA for 6 hours, by using the SiRNA Kit (Targefect; Target Systems). The transfecting medium was changed to medium 199 and the lenses transfected with Cy3 dsRNA were counterstained with the nuclear stain Syto-16 for 30 minutes before imaging on a confocal microscope (LSM-510 META; Carl Zeiss Meditec, Dublin, CA), using a 40 × 0.75 water-immersion objective (LWD; Achroplan; Carl Zeiss Meditec).

Lenses transfected with nonsilencing SiRNA or ALDH1A1-specific SiRNA were used after 72 hours of transfection to study the effects of ALDH1A1 silencing on HNE metabolism and oxidative stress-induced opacification. The time point of 72 hours after transfection was predetermined to be the optimum time in terms of ALDH1 silencing and least toxicity.

Measurement of Apoptosis

Apoptosis was determined by quantifying the cytosolic oligonucleosome-bound DNA, by using an ELISA kit (Cell Death Detection ELISA Kit; Roche Diagnostics), according to the manufacturer's instructions, as we described earlier.[12,27] Briefly, the cytosolic fraction (10,000*g* supernatant) of 5000 cells was used as the antigen source in a sandwich ELISA with a primary anti-histone antibody coating on the microtiter plate and secondary anti-DNA antibody coupled to peroxidase. After the substrate was added, the absorbance was recorded at 405 nm with a microplate spectrometer (SpectroCount; Packard Instruments, Meriden, CT).

Protein-HNE Adducts

Cells were harvested, counted, and cytospun on glass slides, and the rat lens epithelium was fixed to a coated glass slide (Cell Tak; BD Biosciences, Lincoln Park, NJ). The slides were fixed with 4% paraformaldehyde for 30 minutes. After the slides were thoroughly washed with Tris-buffered saline (TBS) containing 0.1% Triton X-100, they were incubated with 1:100 diluted normal goat serum for 30 minutes and then incubated with 1:250 diluted anti-HNE antibodies for 1 hour at room temperature. After thorough washing with TBS, the slides were treated with 1:160 diluted secondary antibody (goat anti-rabbit IgG conjugated with FITC) for 45 minutes. After being thoroughly washed with TBS, the slides were mounted and photographs taken with a fluorescence microscope.

Metabolic Studies

Rat lens ($n = 1$) or HLECs (0.8×10^6), 72 hours after transfection with antisense/scrambled- or SiRNA-transfected lenses or two mouse lenses were incubated with 30 nanomoles ^{3}H-HNE (45,000 cpm) in 2.0 mL Krebs-Hensleit (KH) buffer containing 118 mM NaCl, 4.7 mM KCl, 1.25 mM $MgCl_2$, 3.0 mM $CaCl_2$, 1.25 mM KH_2PO_4, 0.5 mM EDTA, 25 mM $NaHCO_3$, and 10 mM glucose (pH 7.4) at 37°C for 30 and 60 minutes, respectively. At the end of the incubation, the medium was centrifuged and ultrafiltered (Amicon Microcon-10 Filter; Millipore Corp., Bedford, MA). The filtrate thus obtained was subjected to HPLC as described later. The metabolites of ^{3}H-HNE were quantified by determining the radioactivity in each fraction, and the individual peaks were analyzed and characterized as described in the Results section.

HPLC Analysis

Radiolabeled HNE and its putative metabolites (1,4-dihydroxynonene mercapturic acid [DHN], 4,9-dihydroxy-2-nonenoic acid [HNA], glutathione [GS]-HNE and GS-DHN) were synthesized as we described earlier.[17,28] The synthetic HNE metabolites were separated by HPLC, essentially as we described earlier,[29] by using a reverse phase C^{18}-column (Beckman Instruments, Inc., Fullerton, CA). The column was pre-equilibrated with solvent A (0.1% aqueous trifluoroacetic acid) at a flow rate of 1 mL/min. The compounds were eluted with a gradient consisting of solvents A and B (100% acetonitrile) at a flow rate of 1 mL/min. The gradient of solvent A to B was established so that B reached 24% in 15 minutes and held for 5 minutes. In an additional 10 minutes, B reached 26% and was held at this value for 5 minutes. Further, in the next 20 minutes, B reached 100%. Fractions were collected, and the radioactivity was measured with a liquid scintillation radioactivity counter (Beckman Instruments). Using this gradient, the GS-conjugates (GS-HNE and GS-DHN together) eluted with a retention time (τ_R) of 23 minutes, whereas DHN, HNA and HNE eluted at 32, 36 and 43 minutes, respectively.

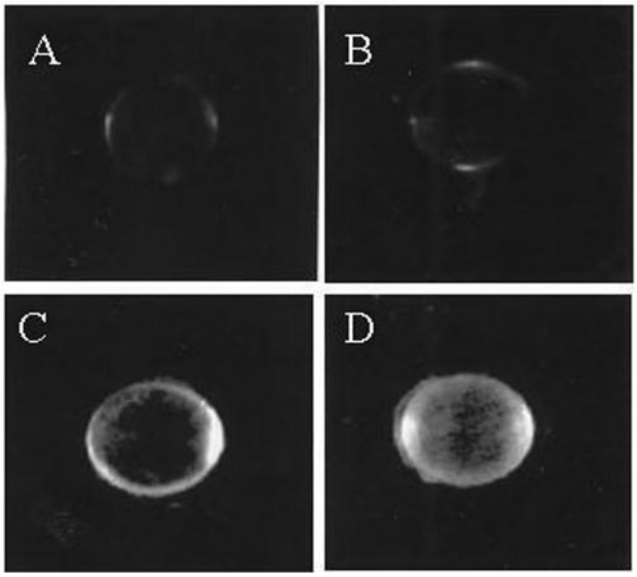

FIGURE 1. Acceleration of oxidation-induced opacification of rat lens by disulfiram. Rat lenses were cultured in medium 199 with or without 1 mM cyanamide for 1 hour. Lenses were then cultured for 48 hours with or without Fenton reagent (100 μM ferrous ammonium sulfate+100 μM H_2O_2+2 mM ADP) and photographed. (**A**) Control; (**B**) control+cyanamide; (**C**) Fenton; and (**D**) Fenton+cyanamide.

Gas Chromatography and Chemical Ionization Mass Spectrometry

DHN, HNA, and HNE were characterized and identified by gas chromatography and chemical ionization-mass spectrometry (GC/CI-MS) as described by us earlier.[17,28,29]

RESULTS

As shown in Figure 1, the control lenses untreated (Fig. 1A) and treated (Fig. 1B) with cyanamide remained clear throughout the time of the experiment. Lenses exposed to Fenton reaction (Fig. 1C) developed significant opacification, which was more pronounced in the cyanamide-treated lens (Fig. 1D). Results of disulfiram-treated lens were same (data not shown). The epithelium was removed and processed for protein-HNE as described in the experimental procedures. Figures 2A, 2C, and 2E show the phase-contrast photographs of the epithelium obtained from lenses treated with the Fenton reagent, disulfiram + the Fenton reagent, and cyanamide + the Fenton reagent, respectively. Figures 2B, 2D, and 2F are the fluorescent images of 2A, 2C and 2F, respectively. It should be noted that at an exposure time of 0 second, 2D and 2F showed intense fluorescence compared with 2B. The inset in 2B shows the fluorescent image at an exposure time of 20 seconds. These results demonstrate that there were higher levels of protein-HNE adducts formed in cyanamide/disulfiram-treated lenses

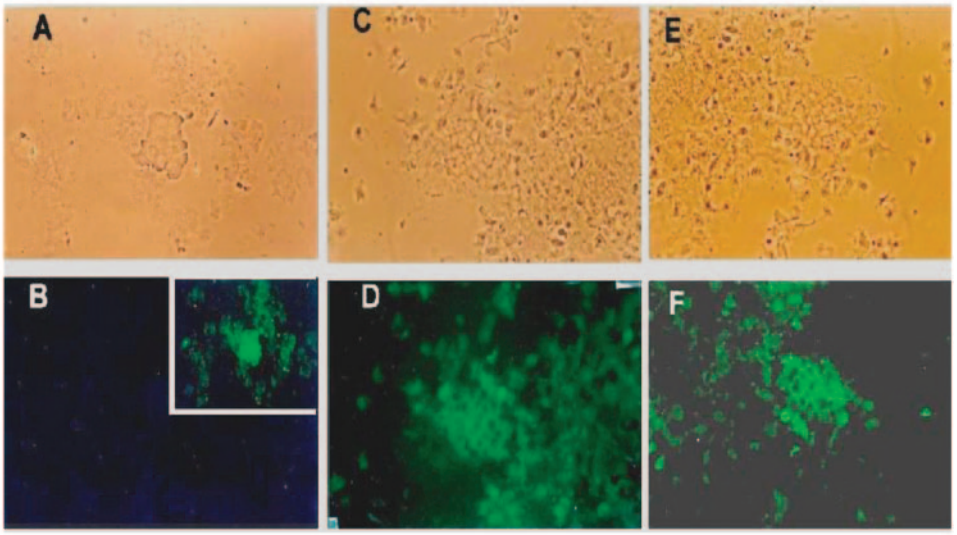

FIGURE 2. Oxidation-induced protein-HNE adduct formation after disulfiram or cyanamide treatment in rat lenses. Epithelia of the disulfiram/cyanamide-treated rat lenses exposed to the Fenton reagent were fixed to coated glass slides, fixed with 4% formaldehyde, and processed to visualize protein-HNE adducts, by using anti-HNE primary antibodies and FITC-conjugated secondary antibodies, as described in experimental procedures and photographed using a fluorescence microscope with an FITC filter. (**A**, **B**) Fenton (phase-contrast and FITC, 0-second exposure, respectively; *inset*: 20-second exposure). (**C**, **D**) Fenton+disulfiram (phase-contrast and FITC, 0-second exposure, respectively); (**E**, **F**) Fenton+cyanamide (phase-contrast and FITC, 0-second exposure, respectively).

exposed to oxidative stress than in those exposed to oxidative stress alone, suggesting that ALDH may play a crucial role in the maintenance of lens clarity under oxidative conditions.

Involvement of ALDH3A1 in the Oxidation of HNE in Mouse Lens and HLECs

The efficiency of SiRNA transfection was evaluated by transfecting Cy3-labeled 23-bp dsRNA in HLECs (Targfectamine; Targeting Systems). Figure 3 shows efficient transfection of SiRNA in these cells, as evident from the red fluorescence due to Cy3 inside the cells. To ascertain the role of ALDH3A1 in protecting against HNE oxidation and HNE-induced cytotoxicity in human lens, we silenced the ALDH3A1 in the HLECs by using either the SiRNA or antisense RNA specific for ALDH3A1, as described in the Methods section. After 72 hours of transfection, cells were evaluated for (1) metabolism of ^{3}H-HNE and (2) HNE or Fenton reagent-induced apoptosis. We did not observe any significant difference in the ability of the experimental and control HLECs to oxidize ^{3}H-HNE (Fig. 4), or in their susceptibility to HNE- or Fenton reaction-induced apoptosis (data not shown). We next compared ALDH3A1-null and wild-type mice. The mouse lenses were assessed for ^{3}H-HNE metabolism and HNE-induced opacification, as described for rat lenses. The results revealed that there was a negligible difference in the levels of ^{3}H-HNA formed (Fig. 4) and in the degree of opacification induced by HNE in the ALDH3A1-knockout versus wild-type mouse lenses (Fig. 5). These results suggest that ALDH3A1 either may not play a significant role in HNE detoxification at the concentrations studied or is virtually absent in the HLECs. RT-PCR using specific primers failed to reveal ALDH3A1, indicating its absence in HLECs (data not shown).

Involvement of ALDH1A1 in HNE-Induced Toxicity in HLECs

Cells were transfected with the scrambled or ALDH1A1-specific antisense oligonucleotide sequences. The levels of ALDH1A1 transcript were monitored at different times after transfection by performing RT-PCR with specific primers. We observed a 60% decrease in ALDH1A1 mRNA with 100 μM antisense oligonucleotide at 72 hours after transfection (Fig. 6A). Results obtained with ALDH1A1-specific antisense were further confirmed by silencing ALDH1A1 with its specific SiRNA. Cells were transfected with 100 μM SiRNA and the transcript level was checked 48 and 72 hours after transfection by RT-PCR, using the specific primers described in the Methods section. Figure 6B, a representative of three experiments, shows that ALDH1A1-specific SiRNA significantly lowered the level of ALDH1A1 mRNA. Control and experimental cells were used to determine the HNE-detoxifying capacity and effect of ALDH1A1 silencing on the oxidative stress-induced cytotoxicity in terms of cell viability and apoptosis. Our results demonstrate that transfecting the cells with antisense or SiRNA decreased the oxidation of ^{3}H-HNE to ^{3}H-HNA by 35% to 40% (Fig. 6C), without a concomitant increase in the glutathionyl conjugate of HNE (data not shown). A parallel increase in apoptosis with a concomitant decrease in cell viability was observed in the cells transfected with ALDH1A1-specific SiRNA or antisense oligonucleotide, compared to the untransfected or scrambled oligonucleotide-transfected cells (Fig. 6C). HNE, be-

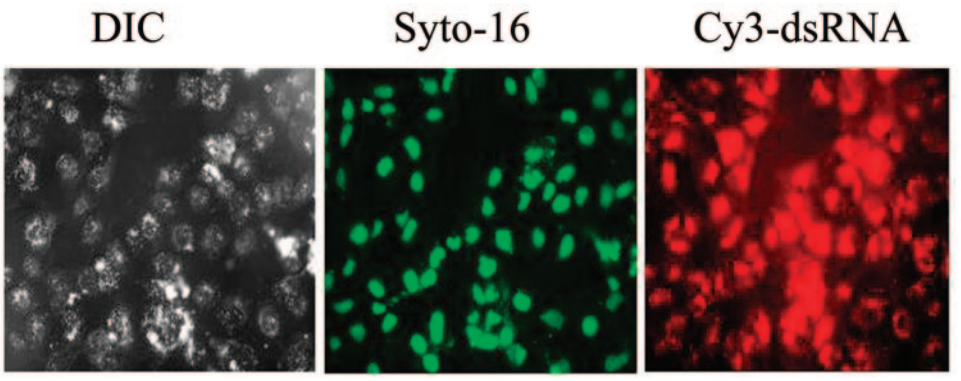

FIGURE 3. HLECs transfection with Cy3-labeled dsRNA. Cells were transfected with 100 picomoles of Cy3-labeled, 23-bp dsRNA for 2 hours using an SiRNA transfection kit. Cells were costained with the Syto-16 nuclear stain and photographed using a fluorescence microscope. The cells were visualized by differential interference contrast (DIC) and in the same field. Cy3 and Syto-16 were visualized using FRITC and FITC filter sets, respectively.

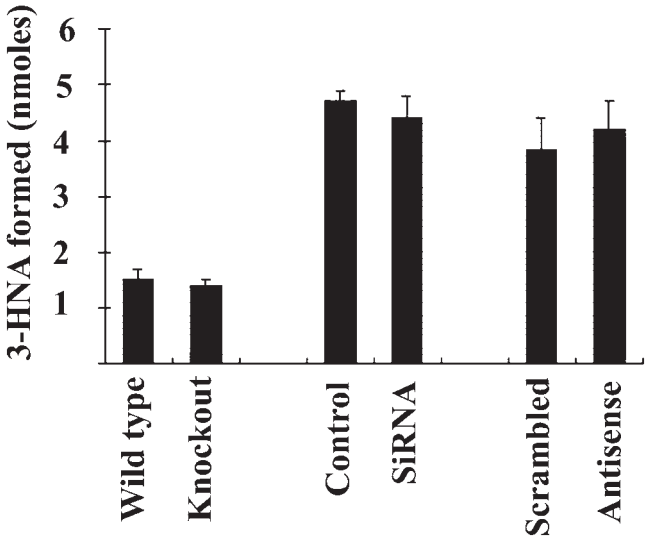

FIGURE 4. Role of ALDH3A1 in HNE oxidation. HNE metabolism was performed in the lenses from ALDH3A1 knockout (KO) and wild-type (WT) mice or ALDH3A1-specific antisense/SiRNA-transfected HLECs. Two mouse lenses or 0.8×10^6 HLECs were incubated with 30 nanomoles of ^{3}H-HNE for 30 minutes, the medium was then ultrafiltered and the metabolites separated. Fractions (1.0 mL) were collected and radioactive counts measured. Values represent nanomoles ^{3}H-HNA formed/two lens or/0.8×10^6 HLECs.

ing a highly reactive aldehyde, readily reacts with proteins and forms protein-HNE adducts. Thus, generation of protein-HNE adduct can be used as a marker to measure HNE-induced toxicity. Therefore, we investigated the effect of ALDH1A1 silencing on the oxidative stress-induced protein-HNE adduct formation in HLECs. Cells were first transfected with 100 μM ALDH1A1-specific antisense oligonucleotide, and 72 hours after transfection the cells were treated with the Fenton reagent. HLECs transfected with ALDH1A1 antisense oligonucleotide and subjected to the Fenton reaction formed significant more protein-HNE adducts than HLECs transfected with scrambled oligonucleotide and treated with the Fenton reagent (Fig. 7).

Involvement of ALDH1A1 in Oxidation and HNE-Induced Toxicity in Rat Lens

Cy3-labeled SiRNA successfully entered through the capsule into the lens epithelium (Fig. 8A; red fluorescence). The green fluorescence represents the Sito-16-stained nuclei of the epithelial cells. The lenses were transfected with 100 μM ALDH1A1-specific SiRNA and exposed to the Fenton reagent. Rat lens cultured without any treatment remained clear for the duration of the experiment (96 hours); however, Fenton reagent-exposed lenses displayed opacification that was signifi-

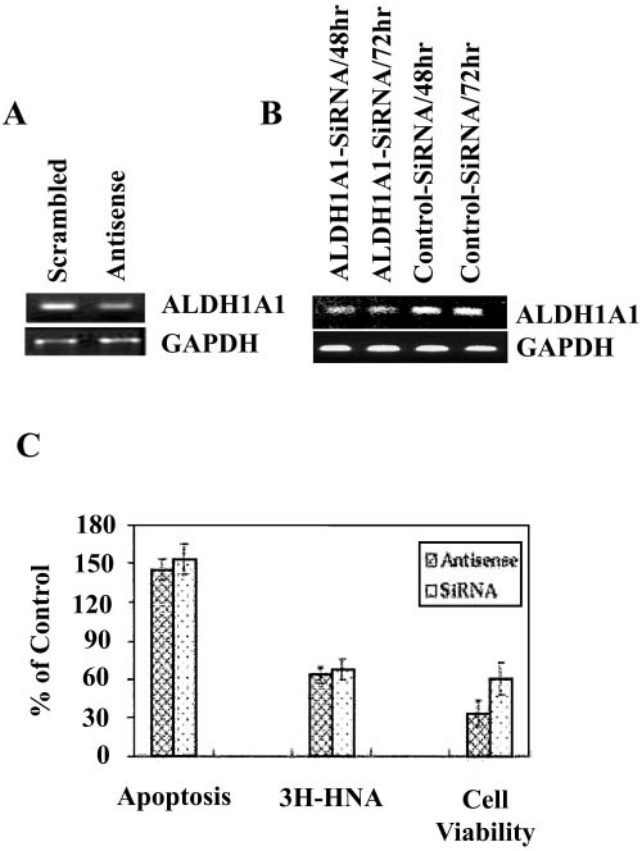

FIGURE 6. Effect of ALDH1A1 ablation on HNE metabolism in HLECs. Decreased levels of ALDH1A1 RNA by (**A**) antisense or (**B**) SiRNA was confirmed by RT-PCR with 2.5 mg of total RNA isolated from either transfected or untransfected HLECs. GAPDH was used as an internal control. (**C**) HLECs transfected with scrambled or ALDH1A-specific antisense/SiRNA were exposed to 50 μM HNE for 3 hours. Cell viability was measured by a trypan blue exclusion assay and apoptosis by ELISA. The ability of the cells to oxidize ^{3}H-HNE to ^{3}H-HNA was determined by incubating the cells (0.8×10^6) with ^{3}H-HNE (30 nanomoles) for 30 minutes and separating the metabolites by HPLC.

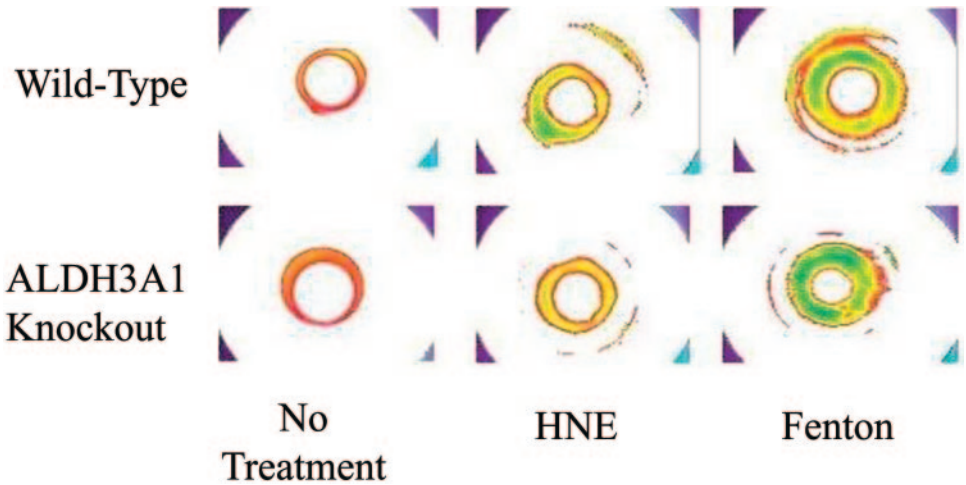

FIGURE 5. Role of ALDH3A1 in oxidation/HNE-induced toxicity. Lenses from wild-type and ALDH3A1 knockout mice were cultured in medium 199 and exposed to 100 μM HNE or Fenton reagent for 48 hours. Bright-field pictures were taken using an inverted microscope with a 4× objective.

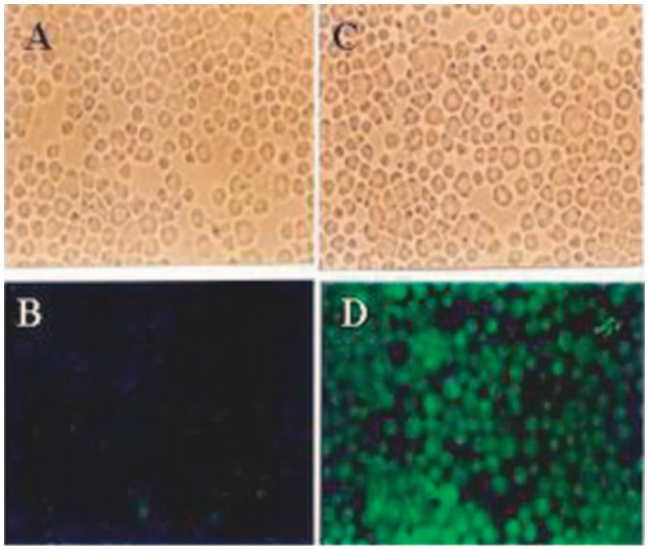

FIGURE 7. Increase in protein-HNE adducts in ALDH1A1-suppressed HLECs exposed to the Fenton reagent. HLECs transfected with ALDH1A1-specific antisense or scrambled oligonucleotides were exposed to the Fenton reagent for 16 hours. The cells were cytospun on glass slides, fixed with 4% formaldehyde, and processed to visualize protein-HNE adducts using anti-HNE primary antibodies and FITC-conjugated secondary antibodies as described in the experimental procedures and photographed (20 seconds exposure) using a fluorescence microscope with an FITC filter. (**A**, **C**) Phase-contrast micrographs of the scrambled and antisense-transfected HLECs, respectively; (**B**, **D**) fluorescent micrographs of (**A**, **C**), respectively.

cantly increased in the lenses transfected with ALDH1A1-specific SiRNA and exposed to Fenton reagent (Fig. 8B). The lenses transfected with ALDH1A1-specific SiRNA showed an ~40% decrease in ALDH1A1 RNA (Fig. 9A) and displayed a proportional decrease in the formation of HNA compared with the lenses transfected with nonsilencing RNA (Fig. 9B). The efficiency of the lenses transfected with ALDH1A1-specific SiRNA in oxidizing ^{3}H-HNE decreased, compared with those transfected with nonsilencing SiRNA (as assessed by the levels of ^{3}H-HNA formed; 5.15 ± 0.52 nanomoles vs. 8.97 ± 1.02 nanomoles). Levels of unmetabolized ^{3}H-HNE remaining in the nonsilencing SiRNA–transfected lens samples were 6.72 ± 0.87 nanomoles, whereas in those transfected with ALDH1A1-specific SiRNA the levels increased to 11.64 ± 2.13 nanomoles.

DISCUSSION

HNE is metabolized by various routes in nonocular tissues[13–16]: (1) oxidation to acid, HNA; (2) reduction to alcohol, DHN; or (3) conjugation with glutathione to form glutathionyl HNE, which can be further reduced to glutathionyl DHN. The process of conjugation to glutathione is carried out by the enzyme glutathione *S*-transferase (GST). The lens contains 5 to 7 mM GSH, and though conjugation with GSH may represent a significant metabolic fate of HNE, it has been suggested that this pathway of conjugative metabolism in liver becomes saturated at fairly low concentrations of HNE. In addition, it has been suggested that if hepatocellular stores of GSH are severely depleted, metabolism of HNE by this pathway may be compromised.[30] Furthermore, it has been reported that α,β-carbonyls act not only as substrates for specific GSTs, but also as potent

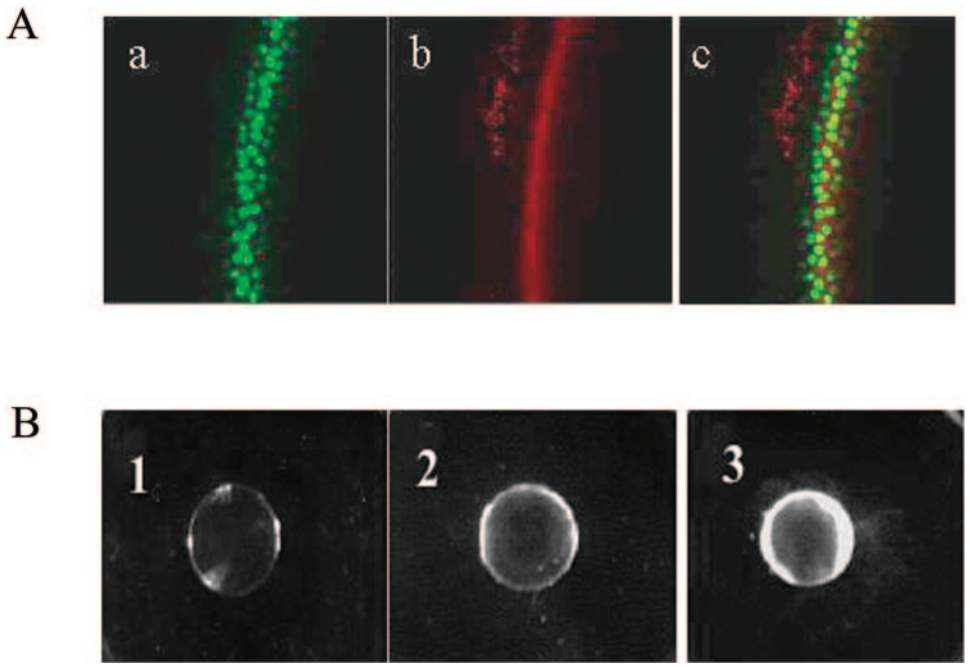

FIGURE 8. (**A**) Transfection of rat lens with labeled Cy3 dsRNA. Lenses were transfected with Cy3 dsRNA for 6 hours; the transfection medium was then changed to medium 199 and cultured overnight. The lens was counterstained with the Syto-16 nuclear stain for 30 minutes before imaging with a confocal microscope equipped with a 40 × 0.75 water-immersion objective. (**Aa**) Image of the lens showing stained nuclei (*green* fluorescence) in the epithelium; (**Ab**) image of the lens showing the presence of Cy3dsRNA (*red* fluorescence) in the epithelium; (**Ac**) images (**Aa**) and (**Ab**) superimposed. (**B**) Effect of ALDH1A1 silencing on oxidative stress-induced lens opacification. The lenses were transfected with nonsilencing or ALDH1A1-specific SiRNA as described in experimental procedures. Seventy-two hours after transfection, lenses were cultured in medium 199 and exposed to the Fenton reagent for 48 hours. (**B1**) lens transfected with nonsilencing SiRNA; (**B2**) lens transfected with nonsilencing SiRNA and treated with the Fenton reagent; (**B3**) lens transfected with ALDH1A1-specific SiRNA and treated with the Fenton reagent.

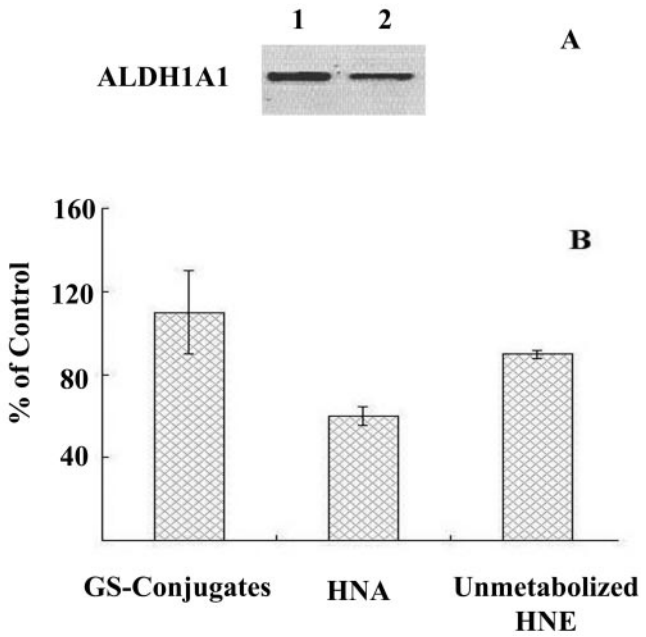

FIGURE 9. Effect of ALDH1A1 silencing on HNA formation in rat lens. (**A**) Soluble proteins were obtained from nonsilencing SiRNA-transfected and ALDH1A1-specific SiRNA-transfected rat lenses after 72 hours of transfection. Ten micrograms of protein each in *lane 1* and *lane 2* was subjected to Western blot analysis with anti-human liver AlDH1A1 antibodies. Anti-rabbit IgG linked to horseradish peroxidase was used as the secondary antibody. The bands were visualized with chemiluminescence. (**B**) Seventy-two hours after transfection, each lens was incubated with 30 nanomoles of ^{3}H-HNE for 60 minutes, the medium was then ultrafiltered and the metabolites separated by HPLC. Fractions (1.0 mL) were collected and radioactive counts measured. The GS-conjugates (GS-^{3}H-HNE+GS-^{3}H-DHN) co-eluted. Formation of the oxidation product (^{3}H-HNA), catalyzed by ALDH1A1, decreased. Data are the amount of metabolites formed by the ALDH1A1-specific SiRNA-transfected lens as a percentage of that formed by the nonsilencing SiRNA control.

inhibitors of these enzymes.[31] It therefore appears that under conditions in which GST or GSH becomes rate limiting, the oxidative and reductive pathways play an effective compensatory role. Aldose reductase catalyzes the NADPH-dependent reduction of HNE and its conjugate, GS-HNE.[32–34] We have shown earlier that aldose reductase preferentially reduces GS-HNE compared with HNE and negligible levels of DHN are formed in HLECs and rat lens.[17] This could have profound physiological relevance. Reduction of GS-HNE may provide an efficient means for attenuating the reactivity of this adduct. It has been shown that the unreduced GSH conjugates of several unsaturated aldehydes are toxic. For example, GS-acrolein is nephrotoxic,[35] the conjugates of hexenal and 2,6-nonadienal induce DNA damage,[36] and glutathionyl propionaldehyde is a potent stimulator of oxygen radical formation.[37] It has been shown that oxidation of unsaturated aldehydes is mediated by NADPH-dependent carbonyl reductases.[38] Although a poor substrate for reduction by carbonyl reductase (K_m = 6 mM), HNE is oxidized by this enzyme (K_m = 60 μM). However, the presence of carbonyl reductase has not been reported in the lens. ALDH represents a significant route for the metabolism of HNE in the liver.[3,39] Both human and bovine lenses contain high levels of ALDH activity.[40–44] Our studies have also shown that a major portion of HNE metabolism in the lens occurs through oxidation.[17] As was evident in the present study, this is a crucial metabolic route for HNE, because inhibition of this pathway increases toxicity in the lens/HLECs. Figure 1 shows that inclusion of the ALDH inhibitors in the culture medium, increases the susceptibility of the lenses to oxidation-induced opacification. In addition, our results in Figure 2 demonstrate that oxidation-induced opacification of the lens is associated with increased formation of protein-HNE adduct, suggesting HNE as a mediator of oxidation-induced cataractogenesis.

Extensive studies related to the structure and function of various ALDH isozymes in nonocular tissues have been performed; however, the role of these isozymes in the lens is still not clear. Further, there has been some discrepancy as to which ALDH isozyme is responsible for the detoxification of HNE. Townsend et al.[45] reported that overexpression of human ALDH3A1 and not human ALDH1A1 provides protection against HNE toxicity by attenuating apoptosis with a simultaneous reduction in protein-HNE adduct formation in V79 cells and a murine macrophage cell line. We did not observe any alteration in HNA formation (Fig. 4) or the extent of oxidative stress-induced lens opacification between ALDH3A1 knockout and wild-type mouse lenses (Fig. 5). Similarly, there was no difference in the levels of HNA formed by the HLECs transfected with ALDH3A1-specific SiRNA/antisense/scrambled oligos (Fig. 4). These results could mean that either ALDH3A1 has a high K_m with HNE or the ALDH3A1 expression in the lens is negligible. Indeed, the $K_{m\text{-HNE}}$ for ALDH3A1 is ~ 20-fold higher than that for ALDH1A1.[46] King et al.[23] reported the presence of both ALDH1A1 and -3A1 in the human lens epithelium, using immunohistochemistry with polyclonal antibodies. However, on purification of these isozymes from human lens they observed abundance of ALDH1A1 and negligible ALDH3A1 activity and protein.[22] Our RT-PCR studies demonstrate the presence of ALDH1A1 (Figs. 6A, 6B) and a virtual absence of ALDH3A1 in HLECs (data not shown) which is in accordance with the report by King and Holmes.[22] We have shown the virtual absence of ALDH3A1 transcripts in mouse lens.[47] It therefore appears that ALDH1A1 is the preferred ALDH isozyme in the lens. Ablation of ALDH1A1 in HLEC by antisense or SiRNA decreased the capacity of the cell to oxidize HNE, with a concomitant increase in HNE-induced toxicity, as determined by measuring cell viability and apoptosis (Fig. 6C). The transfected cells when exposed to oxidative stress by the Fenton reagent displayed higher levels of protein-HNE adducts than did their corresponding controls (Fig. 7). In the rat lens culture experiments, SiRNA was successfully delivered into the epithelium (Fig. 8A). An approximately 40% ablation of ALDH1A1 in the rat lens epithelium made the lens more susceptible to oxidation-induced opacification (Fig. 8B). Such lenses displayed a proportional decrease in the capacity to oxidize ^{3}H-HNE (Fig. 9B). Our results demonstrate the significance of ALDH1A1 in maintaining the lens clarity against oxidation. Because ALDH1A1 is present mainly in the epithelium and outer cortex,[23] which is the first target of UV exposure in the lens, it has been speculated that this enzyme has a protective role against the toxic LDAs generated under oxidative stress. This interpretation is supported in the present study by the correlation of ALDH1A1 levels with HNE metabolism and susceptibility to oxidative insult. It is intriguing that ALDH1A1, not ALDH3A1, is the principal HNE-detoxifying enzyme in the lens, whereas ALDH3A1 predominates in the mammalian cornea (except for rabbits[48]), which is even more exposed to UV irradiation than the lens (for review, see Refs. 49). The high concentration of ALDH3A1 in the mouse cornea (~50% of the water-soluble protein) and the absence of corneal phenotype detected so far in ALDH3A1-knockout mice[24] warrant further studies on the range of functions for corneal ALDH3A1.

In conclusion, we have shown that oxidative metabolism of HNE is a major detoxification route in the mammalian lens. Inhibiting ALDH with disulfiram or cyanamide causes a decrease in HNE oxidation and an acceleration of oxidative cata-

ractogenesis. In addition, decreasing the level of ALDH1A1, with ALDH1A1-specific antisense oligonucleotide or SiRNA, inhibits HNE oxidation and causes an increase in HNE-induced apoptosis in HLECs, and leads to earlier and greater opacification of rat lens in response to oxidative stress, compared with their respective controls. We conclude that ALDH1A1 plays a crucial role in the detoxification of HNE and probably of other toxic aldehydes in the mammalian lens. Our studies thus show the importance of conducting further studies of the role of ALDH1A1 in oxidative cataractogenesis and suggest the possibility of exploiting this pathway for a therapeutic/preventive approach against cataractogenesis.

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