Published in final edited form as:
Free Radic Biol Med. 2014 March ; 68: 80–86. doi:10.1016/j.freeradbiomed.2013.11.028.

ALDEHYDE DEHYDROGENASE 3A1 PROTECTS AIRWAY EPITHELIAL CELLS FROM CIGARETTE SMOKE-INDUCED DNA DAMAGE AND CYTOTOXICITY

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Abstract

Aldehyde dehydrogenase 3A1 (ALDH3A1), an ALDH superfamily member, catalyzes the oxidation of reactive aldehydes, highly toxic components of cigarette smoke (CS). Even so, the role of ALDH3A1 in CS-induced cytotoxicity and DNA damage has not been examined. Among all of the ALDH superfamily members, ALDH3A1 mRNA levels showed the greatest induction in response to CS extract (CSE) exposure of primary human bronchial epithelial cells (HBECs). ALDH3A1 protein accumulation was accompanied by increased ALDH enzymatic activity in CSE-exposed immortalized HBECs. The effects of overexpression or suppression of ALDH3A1 on CSE-induced cytotoxicity and DNA damage (γH2AX) were evaluated in cultured immortalized HBECs. Enforced expression of ALDH3A1 attenuated cytotoxicity and downregulated γH2AX. siRNA-mediated suppression of ALDH3A1 blocked ALDH enzymatic activity and augmented cytotoxicity in CSE-exposed cells. Our results suggest that the availability of ALDH3A1 is important for cell survival against CSE in HBECs.

Keywords

cigarette smoke; airway epithelial cells; chronic obstructive pulmonary disease; ALDH3A1; reactive aldehydes; DNA damage; cytotoxicity; FANCD2

INTRODUCTION

Cigarette smoke (CS) contains abundant reactive aldehydes [1]. In contrast to reactive oxygen species (ROS), aldehydes are relatively long-lived and highly cell permeable, allowing them to cause distant target effects, including DNA damage, glutathione depletion,

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enzyme inactivation and cell death [2]. The aldehyde dehydrogenase (ALDH) superfamily is composed of $NAD(P)^+$-dependent enzymes which catalyze aldehyde oxidation. To date, nineteen ALDH genes have been identified in the human genome [2]. Several studies have demonstrated that human bronchial epithelial cells (HBECs) and lung tissues obtained from smokers exhibit a marked increase in ALDH3A1 gene and protein expression compared to those from nonsmokers [3–6]. However, the biological relevance of cigarette smoke-induced ALDH3A1 upregulation in HBECs has not been examined.

ALDH3A1 is a 54 kDa protein which is constitutively expressed as a homodimer in various tissues, including cornea, lung, esophagus and stomach [2]. ALDH3A1 gene expression can be induced by various xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin), through aryl hydrocarbon receptor (AHR)- or non-AHR-dependent mechanisms [3–5]. ALDH3A1 catalyzes the oxidation of aliphatic, aromatic, and lipid peroxidation (LPO)-derived aldehydes, including 4-hydroxy-2-nonenal (4-HNE) [2]. ALDH3A1 also attenuates LPO-mediated growth inhibition [6], UV light-induced cytotoxicity [7], ROS-induced protein modification [8], and genotoxin-induced DNA damage and apoptosis [9].

CS exposure induces the DNA damage response (DDR) that is mediated by phosphoinositide 3-kinase related protein kinases (PIKKs) [10–11]. Upon DNA damage, the PIKKs phosphorylate the serine 139 residue of H2AX variant (γH2AX) on chromatin flanking DNA double-strand break (DSB) sites. This phosphorylation has been widely used as a sensitive marker of DNA damage [12–13]. γH2AX is crucial for the subsequent DDR signaling and DNA repair [14]. The DDR signaling and activation of PIKKs also inhibits cyclin-dependent kinase (CDK) activity by activating p53 and protein kinases, such as CHK1 and CHK2. Suppression of CDK activity delays cell cycle progression and allows the cells to repair DNA damage [10, 14–16].

Reactive aldehydes, such as 4-HNE, induce DNA interstrand crosslinks (ICLs) [17] that are detrimental to the cell because they block both transcription and replication through inhibition of DNA double-strand unwinding [18]. The Fanconi anemia (FA) pathway, consisting of 14 complementation groups, plays an important role in ICL repair [18]. FANCD2, a member of the FA complementation group (FANC) counteracts aldehyde-induced DNA damage and cytotoxicity both *in vitro* and *in vivo* [19]. Exposure of immortalized HBECs to CS extract (CSE) downregulates FANCD2 protein expression, a change that is detrimental as FANCD2 is required to protect against CSE-induced cytotoxicity [20].

In the present study, we show that among all of the ALDH isozymes, ALDH3A1 exhibits the greatest induction in response to CSE exposure in primary HBECs, and that this induction is mediated by AHR. CSE-exposed immortalized HBECs exhibit a marked increase in ALDH enzymatic activity. ALDH3A1 overexpression attenuates CSE-induced cytotoxicity and DNA damage. Suppression of ALDH3A1 both obstructs ALDH enzymatic activity and augments cytotoxicity induced by CSE. These data suggest that ALDH3A1 modulates CS-induced cytotoxicity and DNA damage in HBECs.

METHODS

Cell Culture

Primary HBECs were isolated from five nonsmokers and maintained under a protocol approved by the LRRI Institutional Review Board as previously described [21]. HBEC2 cells (immortalized HBECs) were originally generated by Ramirez, *et al.* [22] and maintained as previously described [23]. Experiments were performed in twelve-well Costar

tissue culture plates or p100 dishes (100 mm) at a starting cell density of $10 \times 10^3/cm^2$. Cell counts were performed by an electric particle counter (Beckman Coulter, Indianapolis, IN). Twenty-four h after plating, cells were exposed to various concentrations of CSE for 24 and/or 48 h.

Cell Viability

Cell viability was determined by measuring the reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or the trypan blue assay as previously described [24–25]. MTT absorbance was read at 570 nm. CSE-unexposed cells (0% CSE) with or without siRNA transfection or vector transduction were regarded as 100% viability. The relative cell viability of CSE-exposed cells was determined by the comparison with CSE-unexposed cells with the same treatment (*e.g.*, scrambled control or ALDH3A1 siRNA).

Reagents and Antibodies

Chemicals were obtained from Sigma Chemical (St. Louis, MO) and Calbiochem (La Jolla, CA). Protease inhibitors were obtained from Boehringer Mannheim (St. Louis, MO). Polyvinylidene difluoride membranes were obtained from Bio-Rad (Hercules, CA). ECL Plus was obtained from Amersham (Arlington Heights, IL). Antibodies were obtained from various sources: Anti-ALDH3A1, and anti-AHR primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FANCD2 primary antibodies were from Epitomics (Burlingame, CA); phosphorylation-specific antibody for H2AX were from Cell Signaling (Beverly, MA); anti-β actin was from Sigma Chemical (St. Louis, MO). Secondary antibodies (horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig) were obtained from Santa Cruz Biotechnology. Tissue culture plates were obtained from Corning (Corning, NY).

Preparation of Cigarette Smoke Extract (CSE)

100-mm research cigarettes (3R4F) were purchased from the University of Kentucky. CSE solutions were prepared as previously described [26].

Immunoblotting

Immunoblot analysis was performed as previously described [26]. Equivalent loading was verified by stripping the blot and reprobing with antibodies to β-actin. In Figures 3, 4, and 5, relative protein expression was quantified by densitometry and normalized to the corresponding input control (β-actin) bands. Either empty or scrambled control in the absence of CSE was set to value of 1.0.

Real-time RT-PCR for 19 ALDH Isozymes

A customized PCR-Array kit measuring the 19 known ALDH isozymes and *Actb*, was purchased from Qiagen Inc (Valencia, VA). Quantitative analysis of the PCR data was performed according to the manufacturer's instructions. The PCR data were expressed as relative fold difference compared with *Actb* between CSE-treated and non-treated control cells.

ALDH enzymatic activity assay

This assay monitors the production of NAD(P)H from $NAD(P)^+$ as aldehydes are oxidized by ALDH. Total ALDH activity was measured with some modifications to the method previously described [27]. Harvested cell pellet samples were homogenized in a binding buffer of pH 7.5 (1 M Potassium phosphate monobasic, 1M Potassium phosphate dibasic,

0.5 M EDTA, 14.26 mM β-mercaptoethanol) on ice. Homogenates were sonicated (two cycles of 20-second bursts) and incubate for 20 min on ice, and centrifuged at 100,000*g* for 40 min. The enzymatic reaction in the protein supernatants was initiated and monitored over 5 min by the addition of aldehyde substrate (benzaldehyde) into the reaction buffer containing 0.1 M sodium pyrophosphate, 20 mM $NAD(P)^+$, 20 mM pyrazole, 10% β-mercaptoethanol, and 50 μg of total protein and measured spectrophotometrically by monitoring the production of NAD(P)H (340 nm). The ALDH activity was expressed as nmol of NAD(P)H /mg protein/min.

Establishment of cell lines with ALDH3A1 overexpression

HBEC2 cells were infected with lentiviral vectors (pReceiver, GeneCopoeia, Rockville, MD) to overexpress ALDH3A1 protein according to the manufacturer's instructions. The cells were selected with 25 μg/mL hygromycin for 14 d and all surviving colonies were collected as a pool.

RNA interference

Small interfering RNA (siRNA) for AHR, ALDH3A1, and the corresponding scrambled (control) siRNA were purchased from Applied Biosystems (Carlsbad, CA). Transfection of siRNA was performed using INTERFERin (Polyplus-tranfection Inc, New York, NY) according to the manufacturer's instruction. The targeted sequences were as follows: AHR siRNA (catalog #:1199), 5′-GCAUGAUAGUUUUCCGGCUTT-3′; ALDH3A1 siRNA (catalog #:s1244), 5′-GCAACGACAAGGUGAUUAATT-3′.

Statistical analysis

The comparison of CSE induction of the 19 known ALDH isozymes was based on the log transformation of the difference between CSE-exposed and control cells. One sample Student's unpaired t-tests were performed. For all comparisons involving multiple treatment groups, one-way ANOVA was used to identify treatment effects. *p*-values, based on calculated contrasts, were used to assess the individual treatment effects. The results are expressed graphically as the mean ± SEM.

RESULTS

Primary human bronchial epithelial cells exposed to cigarette smoke extract exhibit a robust increase in ALDH3A1

Primary HBECs isolated from 5 nonsmoking donors were cultured in the presence or absence of 1.5% CSE for 24 h. From among 19 ALDH isozymes, exposure to 1.5% CSE significantly increased expression of ALDH3A1 (103.7-fold), ALDH1A3 (7.8-fold), ALDH3A2 (5.6-fold), ALDH5A1 (3.7-fold), ALDH3B1 (2.5-fold), ALDH2 (2.4-fold), ALDH16A1 (2.0-fold), ALDH9A1 (1.9-fold), and ALDH18A1 (1.9-fold) (Figure 1A). None of the ALDH family isozymes were significantly reduced. ALDH3A1 protein levels were increased at 24 h in cultured primary HBECs isolated from three donors (Figure 1B); however, this increase was not observed in HFL1 (normal human fetal lung fibroblasts) or primary lung fibroblasts (data not shown), suggesting that the CSE-induced increase in ALDH3A1 is specific to HBECs but not to fibroblasts.

Cigarette smoke extract-induced ALDH3A1 protein accumulation is accompanied by increased ALDH activity

We next evaluated the effects of CSE on cytotoxicity and ALDH3A1 protein expression in HBEC2 cells. CSE was cytotoxic to HBEC2 cells in a dose- and time-dependent manner (Figure 2A). Similar to the effects of CSE on ALDH3A1 in primary HBECs, CSE increased

ALDH3A1 protein levels (Figure 2B) but this increase did not occur until 8 h after exposure (data not shown). The effects of 1.5% CSE on ALDH enzymatic activity was determined using benzaldehyde, a substrate metabolized by ALDH3A1. Exposure to CSE significantly increased ALDH activity 9.6-fold. While the ALDH activity could have arisen from the increased expression of other ALDH family members, based upon the extent of expression changes, the main contributor for this induction is most likely to be ALDH3A1.

Cigarette smoke extract induces ALDH3A1 *via* aryl hydrocarbon receptor

ALDH3A1 is a transcriptional target of AHR [28], Pax6, and Oct1 [5]. To determine if CSE could induce ALDH3A1 through AHR alone, HBEC2 cells were transfected with either siRNA targeting AHR or a scrambled control. Suppression of AHR expression using siRNA (Figure 3A) reduced CSE-induced ALDH3A1 expression by 78% (Figure 3B). These data suggest that AHR mediates upregulation of ALDH3A1 in response to CSE exposure.

ALDH3A1 attenuates cigarette smoke extract-induced cytotoxicity, DNA damage, and FANCD2 downregulation

To examine the biological relevance of cigarette smoke-induced ALDH3A1 upregulation, we overexpressed ALDH3A1 in HBEC2 cells using a lentiviral vector and exposed them to various concentrations of CSE for 48 h. When compared to empty vector-exposed cells, ALDH3A1-overexpressing cells were protected from CSE-induced cytotoxicity (Figure 4A) and exhibited reduced DNA damage (as reflected in decreased phosphorylation of H2AX) (Figure 4B).

FANCD2 has been recognized to protect against aldehyde-derived DNA damage [19] and CSE-induced cytotoxicity [20]. Given that FANCD2 is downregulated by CSE [20], we sought to determine whether ALDH3A1 overexpression may influence CSE-induced downregulation of FANCD2 [20]. In these experiments, CSE treatment failed to downregulate FANCD2 expression in ALDH3A1-overexpressing cells (Figure 4C). These data suggest that enforced expression of ALDH3A1 attenuates CS-induced cytotoxicity and DNA damage and that CSE-induced FANCD2 downregulation may be mediated by aldehyde metabolism.

To determine if prior endogenous induction of ALDH3A1 by CSE increased resistance to CSE-induced cytotoxicity, HBEC2 cells were pre-exposed to 0.5% CSE before the cytotoxic effects of higher concentrations were established (Figure 2A). This CSE concentration was selected because it induces ALDH3A1 (Figure 2B). In these studies, prior induction of ALDH3A1 significantly protected against CSE-induced cytotoxicity (Figure 4D). These data suggest that the availability of ALDH3A1 prior to CSE exposure may be an important determinant of cell protection against CSE.

Suppression of ALDH3A1 levels both blocks ALDH enzymatic activity and augments cytotoxicity induced by cigarette smoke extract

To further delineate the role of ALDH3A1 in protecting cells from CSE-induced damage, we examined the cytotoxicity of CSE in HBEC2 cells in which ALDH3A1 expression had been suppressed using siRNA. ALDH3A1 siRNA alone significantly reduced cell viability in the absence of CSE (by 59.7 ± 5.0%, $p < 0.01$). Suppression of ALDH3A1 also significantly increased CSE-induced cytotoxicity (Figure 5A). The CSE effects on cell viability determined by the trypan blue assay were similar to those obtained using the MTT assay (Figure 5A). CSE-induced ALDH3A1 expression was reduced by 70% in the presence of ALDH3A1 siRNA (Figure 5B). However, either mock or scrambled siRNA alone increased ALDH3A1.

ALDH enzymatic activity is increased by CSE (see Figure 2C); however, the question remains whether this is primarily due to induction of ALDH3A1. In HBEC2 cells, siRNA targeting ALDH3A1 prevented the CSE-induced increase in ALDH enzymatic activity (Figure 5C). Identical results were obtained when AHR expression was suppressed using siRNA. These results suggest that CSE-induced ALDH activity is likely primarily dependent on AHR-mediated ALDH3A1 induction. It is also important to note that scrambled siRNA transfection alone increased ALDH activity. When compared to the scrambled control siRNA, either AHR or ALDH3A1 siRNA significantly reduced the ALDH activity in the absence of CSE (Figure 5C).

DISCUSSION

In this study, we show that ALDH3A1 is the most robustly induced isozyme among the ALDH superfamily in CSE-exposed primary HBECs. Our studies demonstrate that CSE robustly upregulates ALDH enzymatic activity in HBECs through a mechanism dependent on AHR-mediated ALDH3A1 expression. Enforced expression of ALDH3A1 attenuates CSE-induced cytotoxicity, DNA damage and FANCD2 downregulation. By contrast, suppression of ALDH3A1 augments CSE-induced cytotoxicity and blocks the ALDH enzymatic activity. These data suggest an important role of ALDH3A1 in airway epithelial cell survival by protecting from DNA damage-induced cell death.

While other ALDH isozymes including ALDH1A3, ALDH2, ALDH3A2, ALDH3B1, ALDH5A1, ALDH19A1, ALDH16A1 and ALDH18A1 were significantly upregulated in CSE-exposed primary HBECs, none of the nineteen ALDH isozymes was downregulated by CSE. Previous human studies have demonstrated that both gene and protein expression of ALDH3A1 are upregulated in airway epithelial cells of smokers relative to those of nonsmokers [29–30]. Patel and colleagues also showed that immunohistochemical expression of ALDH3A1 protein is significantly increased in non-small cell lung cancer cells relative to normal pneumocytes or small cell lung cancer cells [29]. However, a potential role of ALDH3A1 in malignant transformation remains to be determined.

In this study, we determined the effects of CSE on ALDH enzymatic activity in HBECs. We found that CSE markedly increased ALDH activity in immortalized HBEC2 cells. CSE-induced increase in ALDH activity appeared to be attributable to ALDH3A1 given that siRNA targeting of ALDH3A1 completely blocked CSE-induced ALDH enzymatic activity. Interestingly, a previous *in vitro* study using human blood cells (consisting of >99.5% red blood cells) demonstrated that CSE inhibited ALDH enzymatic activity in a dose-dependent manner [31]. Also, in the current study CSE exposure did not increase ALDH3A1 expression in primary human lung fibroblasts (data not shown). When taken together, these results would suggest that the effects of CS on ALDH activity are cell type-specific.

We further investigated the role of ALDH3A1 in modulating the cytotoxic effects of CSE by genetically-manipulating cellular levels of ALDH3A1. We showed that overexpression of ALDH3A1 attenuates CSE-induced cytotoxicity in cultured immortalized HBECs. Prior endogenous induction of ALDH3A1 by pre-exposure to a low (0.5%) concentration of CSE also reduced subsequent CSE-induced cytotoxicity. However, it is known that CS exposure significantly alters expression of numerous genes (*e.g.*, up-regulating multiple genes encoding antioxidant enzymes to scavenge ROS or oxidize aldehydes) in the lung of small animals [32] and in HBECs [33]. We thus speculate that the protective effects of CSE-pretreatment are likely dependent on multiple pathways. However, we think that the availability of ALDH3A1 before CSE exposure is still important for cell survival against CSE because ALDH3A1 overexpression was sufficient to attenuate CSE-induced cytotoxicity. It should be also noted that the transfection procedure alone modestly increases

ALDH3A1 expression and activity, possibly due to cellular stresses caused by transfection. The present data are consistent with previous reports demonstrating that ALDH3A1-transfected cells are more resistant to cytotoxicity by a variety of genotoxins, including hydrogen peroxide, 4-HNE, mitomycin C, etoposide, and ultraviolet light [7, 9]. Notably, ALDH3A1-transfected cells have also been shown to exhibit more resistance to glutathione depletion in response to DNA-damaging agents [9]. In sum, these observations suggest that ALDH3A1 plays a crucial role in cell survival against oxidative stress induced by genotoxins.

The present studies also suggest that ALDH3A1 overexpression is sufficient to protect against CSE-induced DNA damage and FANCD2 downregulation. A previous *in vitro* study in immortalized HBECs demonstrated that FANCD2 overexpression protects against CSE-induced cell death and FANCD2 loss augments the effects of CSE [20]. Moreover, Hays and colleagues showed that exposure to CSE downregulates FANCD2 via translational inhibition [20]. Given that FANCD2 is a key player in the Fanconi anemia pathway involved in repair of ICLs derived from reactive aldehydes [18], the protective effects of ALDH3A1 on DNA damage and cytotoxicity may be, in part, mediated by FANCD2 expression.

Loss of AHR by siRNA transfection markedly suppressed CSE-induced ALDH3A1 upregulation in HBECs. These data suggest that ALDH3A1 upregulation by CSE is primarily mediated by AHR. Although ALDH3A1 can be upregulated through non-AHR dependent pathways, such as Pax6 and Oct1 [4–5], it is unlikely that these pathways contribute significantly to CSE-mediated induction of ALDH3A1. A recent *in vitro* study demonstrated that suppression of AHR levels augments CSE-induced cell death in cultured mouse lung epithelial cells [34]. Rico de Souza and colleagues attributed enhancement of CSE-induced apoptosis to down-regulation of both CuZn superoxide dismutase (CuZnSOD) and mitochondrial SOD expression in AHR-deficient cells [34]. However, the present study suggests that suppression of ALDH3A1 expression due to AHR loss may also contribute to the augmented CSE-induced cell death. ALDH3A1 catalyzes the oxidation of various LPO-derived aldehydes, including 4-HNE [2]. A previous human study demonstrated that 4-HNE levels are elevated in the lungs of smokers with chronic obstructive pulmonary disease (COPD) compared with those of smokers without COPD [35], suggesting a potential role of aldehyde metabolism in the pathogenesis of COPD. Other known preferred substrates of ALDH3A1 are medium-chain length aliphatic and aromatic aldehydes [2], both of which are present in CS [36]. However, the other aldehyde components in CS, including formaldehyde, acrolein, and acetaldehydes, are not oxidized by ALDH3A1 [2]. Accordingly, the protective effects of ALDH3A1 on CSE-induced cytotoxicity are likely associated with detoxification of 4-HNE, medium-chain length aliphatic and aromatic aldehydes. Further studies will be required to determine the effects of ALDH3A1 modulation on CS-induced COPD.

Acknowledgments

This manuscript was supported in part by ALA Biomedical Research Grant: RG-231988-N, Clinical and Translational Science Center 003-2, AG037768-01, 10GRNT3530045 (to TN), HL068111 and ES015482 (to YT), the Tobacco Master Settlement through a cooperative research agreement with the University of New Mexico, DK065988 (to SHR) and EY11490 and EY17963 (VV).

Abbreviation

AHR	aryl hydrocarbon receptor
ALDH	aldehyde dehydrogenase

ALDH3A1 OE	ALDH3A1 overexpression
ANOVA	analysis of variance
ATM	teleangiectasia mutated protein
CDK	cyclin dependent kinase
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
CSE	cigarette smoke extract
DDR	DNA damage response
DSB	DNA double-strand break
FA	Fanconi anemia
FANCD2	a member of the FA complementation group (FANC)
4-HNE	4-hydroxy-2-nonenal
HBEC	human bronchoepithelial cells
ICLs	DNA interstrand crosslinks
LPO	lipid peroxidation
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NS	not significant
PAHs	polycyclic aromatic hydrocarbons
PIKKs	phosphoinositide 3-kinase related protein kinases
ROS	reactive oxygen species
RONS	reactive oxygen/nitrogen species
SEM	standard error of the mean
siRNA	small interfering RNA
SOD	superoxide dismutase
TB	trypan blue
TCDD	dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin

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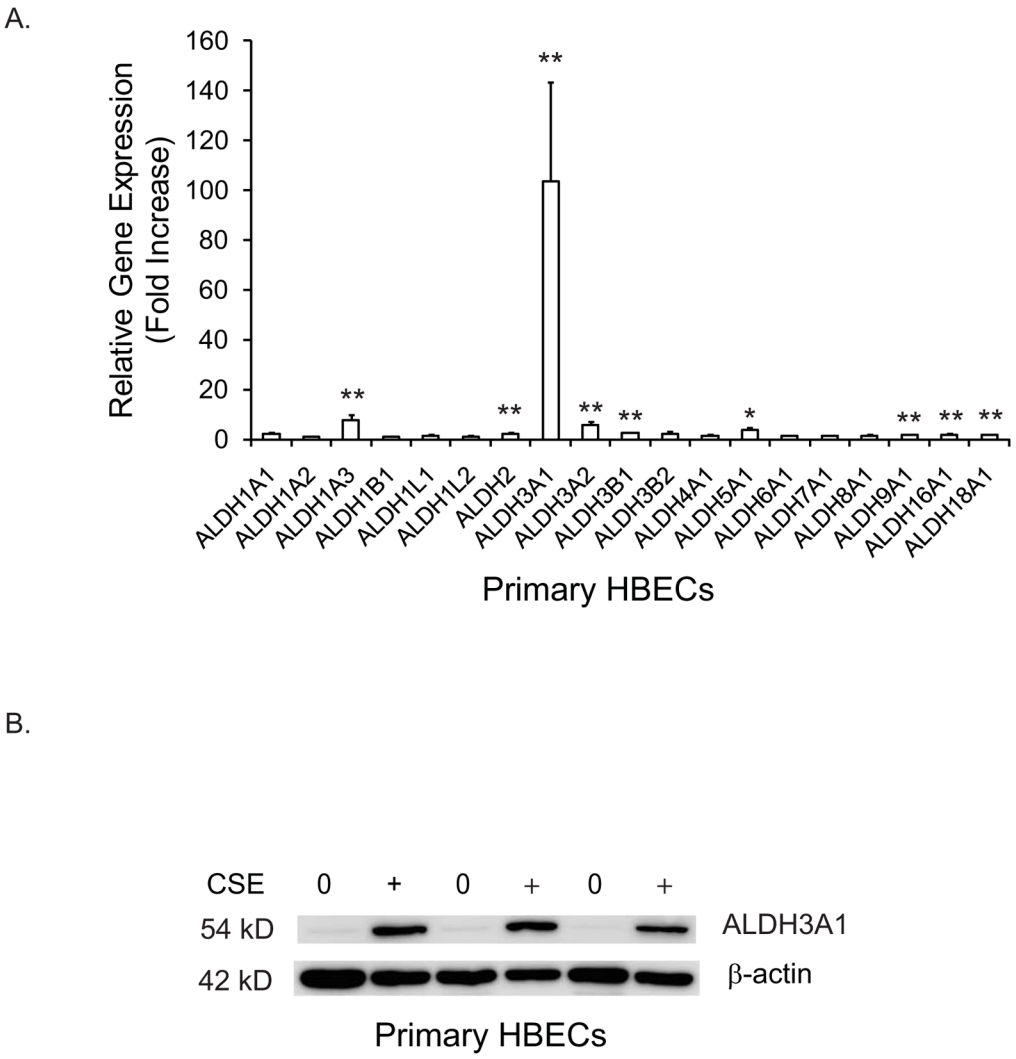


Figure 1. Primary human bronchial epithelial cells exposed to cigarette smoke extract exhibit a robust increase in ALDH3A1

Primary HBEC cells isolated from non-smoking donors were cultured in the absence or presence of 1.5% CSE for 24 h. **A.** Steady-state levels of mRNA of the 19 known isozymes of ALDH were measured by RT-PCR and presented as relative fold difference compared with *Actb* between CSE-treated and non-treated control cells. Data are expressed as mean ± SEM from 5 donors (*, $p < 0.05$; **, $p < 0.01$). **B.** Primary HBECs isolated from three non-smoking donors were cultured in the absence (0) or presence (+) of 1.5% CSE for 24 h. Immunoblot analysis of ALDH3A1 was performed.

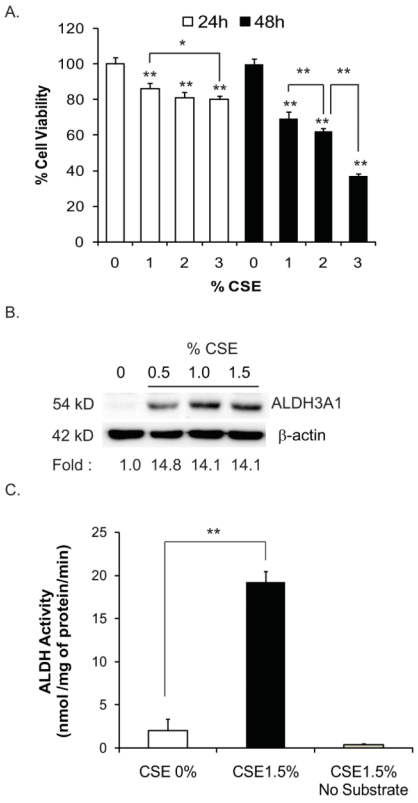


Figure 2. Cigarette smoke extract-induced ALDH3A1 protein accumulation is accompanied by increased ALDH activity

A. HBEC2 cells were cultured in the presence or absence of CSE (0, 1, 2, and 3%) for 24 or 48h. Cell viability was determined by the MTT assay. Data are expressed as mean ± SEM for two independent experiments with three triplicate samples (*, $p < 0.05$; **, $p < 0.01$). **B**. HBEC2 cells were cultured in the absence (0) or presence of CSE (0.5, 1.0, and 1.5%). Immunoblot analysis of ALDH3A1 was performed 24 h thereafter. Data are representative of three experiments. **C**. At the end of a 24 h period of treatment without (CSE 0%) or with 1.5% CSE (CSE 1.5%), ALDH enzymatic activity was measured in HBEC2 cells using benzaldehyde (as a substrate) to initiate the reaction. Enzyme activity was also measured in CSE-treated cells to which no benzaldehyde (CSE 1.5% No substrate) was added to determine background NAD(P)H production. Data are expressed as mean ± SEM from three independent experiments with three triplicate samples (**, $p < 0.01$).

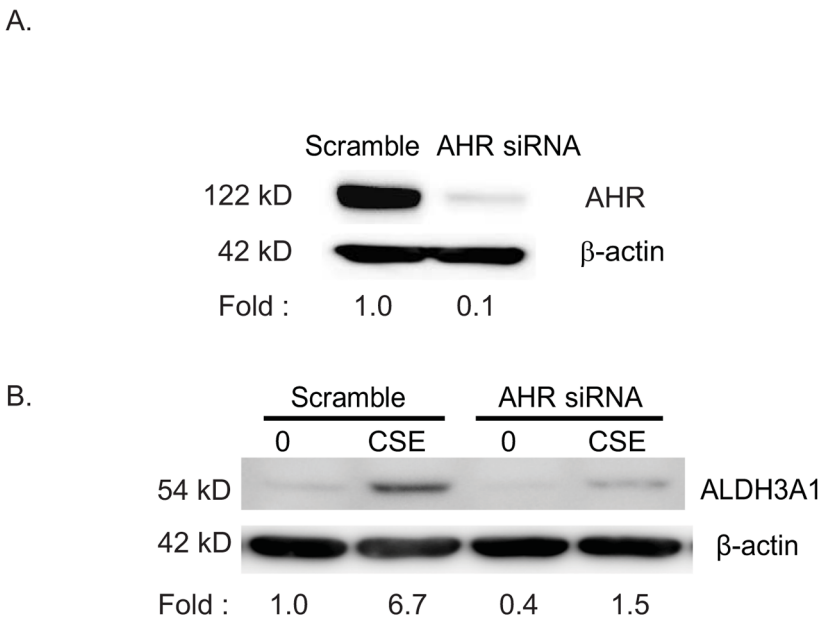
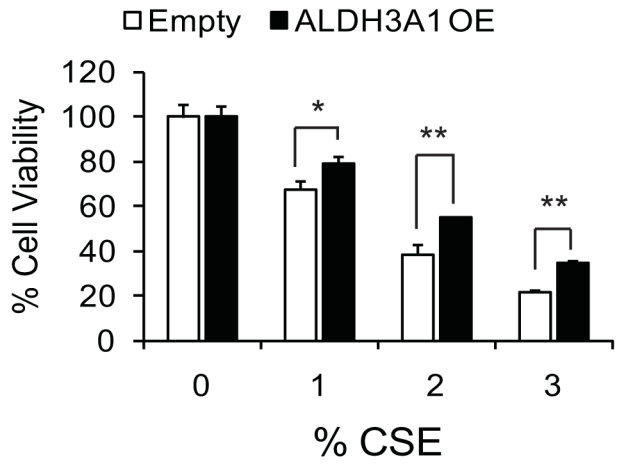


Figure 3. Cigarette smoke extract induces ALDH3A1 *via* aryl hydrocarbon receptor

A. HBEC2 cells were transfected with either siRNA targeting AHR (AHR siRNA) or the scrambled siRNA (Scrambled) as control. Immunoblot analysis of AHR was performed 24 h after transfection. **B.** HBEC2 cells were treated as in A. and then incubated in the absence (0) or presence of 1.5% CSE (CSE). Immunoblot analysis of ALDH3A1 was performed 24 h thereafter. Data are representative of three experiments.

A.



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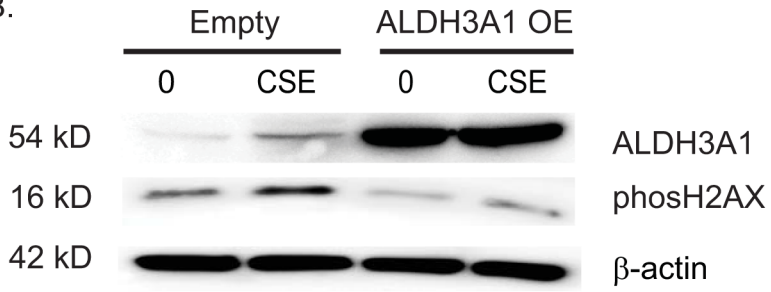
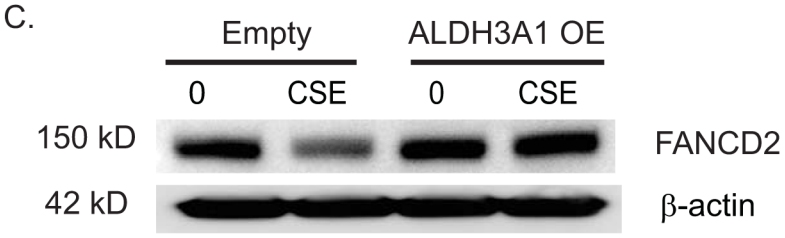


C.



D.

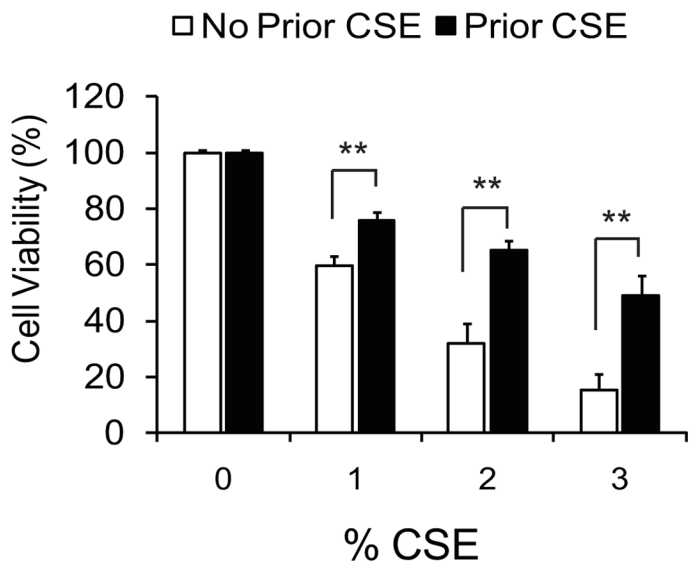


Figure 4. ALDH3A1 attenuates cigarette smoke extract-induced cytotoxicity, DNA damage, and FANCD2 downregulation

HBEC2 cells were transduced with a lentiviral vector (pReceiver) encoding ALDH3A1 cDNA or the empty vector and selected with 25 μg/mL hygromycin. HBEC2 cells treated with empty vector (Empty) or overexpressing ALDH3A1 (ALDH3A1 OE) were cultured in the absence (0%) or presence of CSE (1, 2, and 3%) for 48 h. **A.** Cell viability was determined using the MTT assay at 48 h. Data are expressed as mean ± SEM for three independent experiments (*, $p < 0.05$; **, $p < 0.01$). **B**. Immunoblot analysis of ALDH3A1 and phosphorylated H2AX (phos H2AX) was performed 24 h after treatment without (0) or with 1.5% CSE (CSE). Immunoblotting data are representative of three experiments. **C**. HBEC2 cells were treated as in B. Immunoblot analysis of FANCD2 was performed. Data are representative of three experiments. **D.** HBEC2 cells were pretreated with vehicle (No Prior CSE) or 0.5% CSE (Prior CSE) for 24 h, and then further incubated in the absence (0) or presence of CSE (1, 2, and 3%) for additional 48 h. Cell viability was determined using the MTT assay at 48 h. Cell viability was expressed as a percentage of the absorbance intensity relative to the control cells without (No Prior CSE) or with CSE-pretreatment (Prior CSE) 48 h after culture in the absence of CSE. Data are expressed as mean ± SEM for three independent experiments (**, $p < 0.01$).

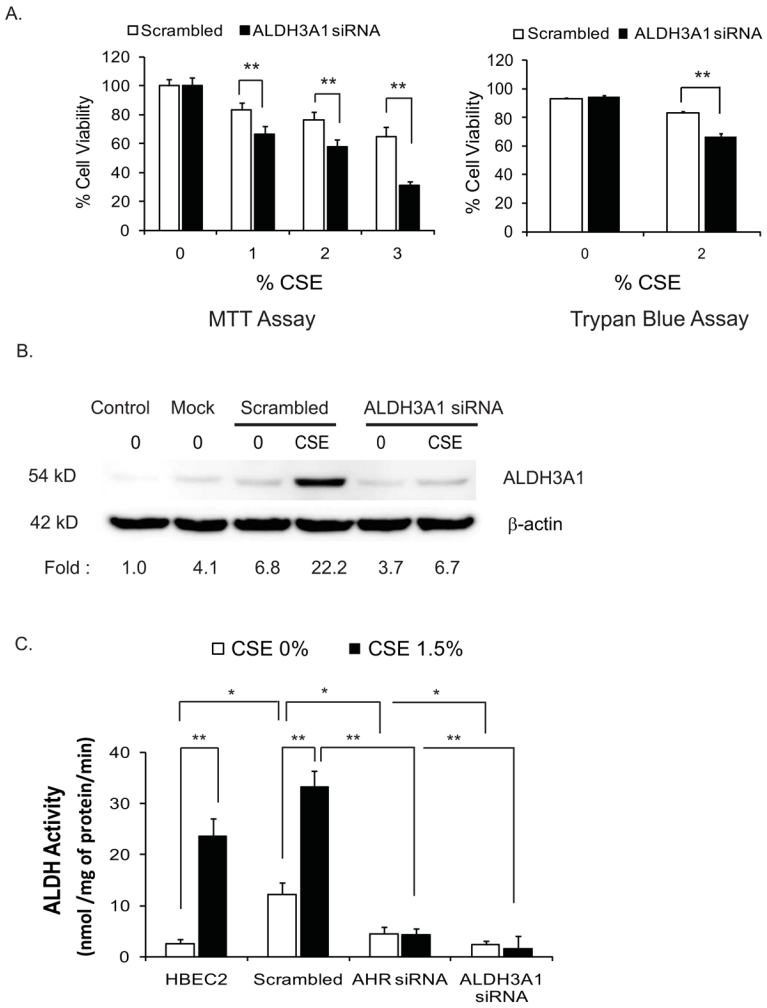


Figure 5. Suppression of ALDH3A1 levels both blocks ALDH enzymatic activity and augments cytotoxicity induced by cigarette smoke extract

A. HBEC2 cells transfected with siRNA targeting ALDH3A1 mRNA (ALDH3A1 siRNA) or the scrambled control (Scrambled) were cultured for 24 h followed by further incubation in the absence (0%) or presence of CSE (1, 2, or 3%). Cell viability was determined using the MTT assay and trypan blue assay (only 2% CSE) at 48 h. Data are expressed as mean ± SEM from two independent experiments with triplicate samples (**, $p < 0.01$). **B**. HBEC2 cells were treated as in A, except for including the control (no treatment) and mock (reagent only). Immunoblot analysis of ALDH3A1 was performed 24 h after treatment without (0) or with 1.5% CSE (CSE). Data are representative of three experiments. **C.** HBEC2 cells were treated as in A. In addition, some HBEC2 cells were not transfected (Control) or transfected with siRNA targeting AHR mRNA (AHR siRNA), ALDH3A1 mRNA (ALDH3A1 siRNA), or the scrambled siRNA (Scrambled). ALDH enzymatic activity was measured spectrophotometrically through the measurement of NAD(P)H, a byproduct of benzaldehyde (substrate) oxidation by ALDH. Data are expressed as mean ± SEM from three independent experiments with three triplicate samples (*, $p < 0.05$; **, $p < 0.01$).