Smoking-Induced Upregulation of AKR1B10 Expression in the Airway Epithelium of Healthy Individuals

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Background: The aldo-keto reductase (AKR) gene superfamily codes for monomeric, soluble reduced nicotinamide adenine dinucleotide phosphate-dependent oxidoreductases that mediate elimination reactions. AKR1B10, an AKR that eliminates retinals, has been observed as upregulated in squamous metaplasia and non-small cell lung cancer and has been suggested as a diagnostic marker specific to tobacco-related carcinogenesis. We hypothesized that upregulation of AKR1B10 expression may be initiated in healthy smokers prior to the development of evidence of lung cancer.

Methods: Expression of AKR1B10 was assessed at the mRNA level using microarrays with TaqMan confirmation in the large airway epithelium (21 healthy nonsmokers, 31 healthy smokers) and small airway epithelium (51 healthy nonsmokers, 58 healthy smokers) obtained by fiberoptic bronchoscopy and brushing.

Results: Compared with healthy nonsmokers, AKR1B10 mRNA levels were significantly upregulated in both large and small airway epithelia of healthy smokers. Consistent with the mRNA data, AKR1B10 protein was significantly upregulated in the airway epithelium of healthy smokers as assessed by Western blot analysis and immunohistochemistry, with AKR1B10 expressed in both differentiated and basal cells. Finally, cigarette smoke extract mediated upregulation of AKR1B10 in airway epithelial cells in vitro, and transfection of AKR1B10 into airway epithelial cells enhanced the conversion of retinal to retinol.

Conclusions: Smoking per se mediates upregulation of AKR1B10 expression in the airway epithelia of healthy smokers with no evidence of lung cancer. In the context of these observations and the link of AKR1B10 to the metabolism of retinals and to lung cancer, the smoking-induced upregulation of AKR1B10 may be an early process in the multiple events leading to lung cancer.

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Abbreviations: 16HBE = 16 human bronchial epithelium; AKR = aldo-keto reductase; CSE = cigarette smoke extract; NSCLC = non-small cell lung cancer; PCR = polymerase chain reaction

The aldo-keto reductase (AKR) superfamily includes a group of monomeric 37-kDa soluble reduced nicotinamide adenine dinucleotide phosphate-dependent oxidoreductases that function in elimination reactions

by modifying carbonyl groups on aldehyde or ketones to form primary or secondary alcohols, which are then conjugated with sulfates or glucuronide for excretion.^{1–6} Like the cytochrome P450 superfamily, the AKRs are classified as a phase 1 drug, xenobiotic and carcinogen-metabolizing enzymes.⁷ One of the

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many members of the AKR family is AKR1B10, an enzyme with a preference for eliminating retinals.⁸⁻¹⁰ AKR1B10 is normally expressed in the small intestine and colon, with low-level expression in the liver but not in the lung.¹¹ However, a number of studies have demonstrated that it is overexpressed in smokers with non-small cell lung cancer (NSCLC), suggesting that AKR1B10 may be a useful marker for NSCLC.^{12,13} In the context that AKR1B10 has a high catalytic efficiency for the reduction of retinals and that a deficiency of retinoic acid is associated with airway epithelial squamous metaplasia and epithelial-mesenchymal transition, increased airway epithelial expression of AKR1B10 may play an important role in the pathogenesis of lung cancer.^{14,15}

Based on these considerations and with the knowledge that most lung cancers occur in persons with a history of cigarette smoking, we hypothesized that cigarette smoking per se upregulates airway epithelial expression of AKR1B10, with increased AKR1B10 upregulation occurring in healthy individuals before any evidence of lung cancer. Using microarray, TaqMan polymerase chain reaction (PCR), Western blot analysis, and immunohistochemistry analysis of AKR1B10 expression in the airway epithelium of healthy nonsmokers and healthy smokers, the data demonstrate a dramatic upregulation of AKR1B10 in both large and small airway epithelia of healthy smokers. Consistent with the observations, cigarette smoke extract (CSE) induces the expression of AKR1B10 in vitro in human airway epithelium in culture, and transfection of AKR1B10 into airway epithelial cells enhances the conversion of retinal to retinol. Together, the data demonstrate that smoking per se initiates on the expression of AKR1B10 in the airway epithelium, suggesting that increased AKR1B10 expression may be an early event in the progression of the airway epithelium to lung cancer.

MATERIALS AND METHODS

Study Population

All subjects were recruited through advertisements in local newspapers, on electronic bulletin boards, and through an ongoing program of free spirometry screening. The evaluation of all individuals was performed at the Department of Genetic Medicine Clinical Research Facility under the auspices of the Weill Cornell National Institutes of Health Clinical Translational Science Center (New York, NY), using Institutional Review Board-approved clinical protocols. Nonsmokers and smokers were determined to be healthy based on standard history, physical examination, CBC count, coagulation studies, liver function tests, HIV1 serology, α_1 -antitrypsin levels, urine studies, chest radiography, ECG, and pulmonary function tests. Current smoking status was evaluated based on history (pack-years), venous carboxyhemoglobin levels, and urine analysis for nicotine metabolites. The inclusion criteria

for healthy nonsmokers were a history of never smoking and normal physical examination, lung function, and chest radiograph, with smoking-related blood and urine parameters within the nonsmoker range. The criteria for healthy smokers were current smoking history and normal physical examination, lung function, and chest radiograph, with smoking-related blood and urine parameters consistent with that of a current smoker.^{16,17}

Sampling Airway Epithelium

Large airway and small airway epithelium brushes were collected using fiberoptic bronchoscopy, as previously described.^{18,19} Briefly, after mild sedation with meperidine and midazolam and routine anesthesia of the vocal cords and bronchial airways with topical lidocaine, a fiberoptic bronchoscope (Pentax EB-1530T3; Pentax Imaging Co; Golden, CO) was taken proximal to the opening of a desired lobar bronchus. Large and small airway epithelial cells were collected from the third- to fourth-order and 10th- to 12th-order bronchi separately, by gently gliding the brush back and forth five to 10 times in eight to 10 different locations in the same general area. Cells were detached from the brush by flicking and immediately transferred into aliquots of ice-cold LHC8 medium (Gibco; Grand Island, NY). Total cell number was counted on a hemocytometer, and cell viability was estimated by trypan blue exclusion and expressed as a percentage of the total cells recovered. About 3×10^6 to 5×10^6 cells were processed immediately for RNA extraction.

Morphology Characterization of Airway Epithelial Cells

To quantify the percentage of epithelial and inflammatory cells and the proportions of ciliated, secretory, undifferentiated, and basal epithelial cells, aliquots of 10^4 cells per slide were prepared by centrifugation (Cytospin 11; Shandon Instruments; Pittsburgh, PA) and stained with Diff-Quik (Dade Behring; Newark, DE). The quantification of the percentage of different cell types was carried out blindly by a well-trained observer. Fields were randomly chosen, and a total of 300 cells were counted. The morphologic criteria for each type of cell were strictly followed. Important features of the cells are as follows: for ciliated cells, cilia, dense plate, and cytoplasm under cilia; for secretory cells, many secretory granules, some with translucent contents, and relatively big in size; for undifferentiated cells, a long spindle shape, with some presenting as round cells with a significant amount of cytoplasm; for basal cells, compact nuclei and minimal, but visible cytoplasm present as single cells or in groups.

RNA Extraction, Microarray Processing, and Data Analysis

Analysis was performed using HG-U133 Plus 2.0 microarray (Affymetrix; Santa Clara, CA) (54,675 probe sets representing approximately 47,000 full-length human gene transcripts) and associated protocols. Total RNA was extracted using a modified version of the TRIzol method (Invitrogen; Carlsbad, CA), followed by RNeasy (Qiagen; Valencia, CA) to remove residual DNA. RNA samples were stored in RNA Secure (Ambion Inc; Austin, TX) at -80°C until further processing. An aliquot of each RNA sample was run on a bioanalyzer (Agilent Technologies; Palo Alto, CA) to visualize and quantify the degree of RNA integrity. The concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE).

Double-stranded cDNA was synthesized from 1 to 2 μg of total RNA using the GeneChip One-Cycle cDNA Synthesis Kit followed by cleanup with GeneChip Sample Cleanup Module, in vitro transcription reaction using the GeneChip IVT Labeling Kit, and cleanup and quantification of the biotin-labeled cRNA yield by spectrophotometric analysis (all kits from Affymetrix).

Hybridizations to test chips and to the microarrays were performed according to Affymetrix protocols, and microarrays were processed by the Affymetrix fluidics station and scanned with the Affymetrix GeneArray Scanner 2500. Overall microarray quality was verified by the following criteria: (1) RNA Integrity Number ≥ 7.0 ; (2) 3'/5' ratio for glyceraldehyde-3-phosphate dehydrogenase ≤ 3 ; and (3) scaling factor ≤ 10.0 .²⁰ Captured images were processed using the Microarray Suite version 5.0 software algorithm (Affymetrix), which takes into account the perfect-match and mismatch probes. The data were normalized using GeneSpring, version 7.3 software (Agilent Technologies) per array by dividing the raw data by the 50th percentile of all measurements.

TaqMan Real-Time PCR

To confirm the gene expression of AKR1B10 in large and small airway epithelia, TaqMan real-time PCR was carried out on a random selection of RNA samples from large airways (14 healthy nonsmokers and 14 healthy smokers) and small airways (18 healthy nonsmokers and 18 healthy smokers) that also had been assessed with the HG-U133 Plus 2.0 array. The cDNA was synthesized from 2 μg of RNA in a 100 μL reaction volume using the TaqMan Reverse Transcriptase Reaction kit (Applied Biosystems; Foster City, CA) with random hexamers as primers. Dilutions of 1:10 and 1:100 were made from each sample, and triplicate wells were run for each dilution. TaqMan real-time PCR reactions were carried out using premade gene expression assays for the AKR1B10 gene from Applied Biosystems, and 2 μL of cDNA were used in each 25 μL reaction volume. The endogenous control was 18S rRNA, and relative expression levels were determined using the $\Delta\Delta C_t$ method (Applied Biosystems), with the average value for the nonsmokers as the calibrator. The PCR reactions were run in the Applied Biosystems Sequence Detection System 7500.

Western Blot Analysis

Western blot analysis was used to quantify AKR1B10 protein expression in large and small airway epithelia from healthy nonsmokers and healthy smokers. The whole cells were suspended in RBC lysis buffer (eBioscience; San Diego, CA) for 10 min. Cells then were collected by centrifugation at 400g for 5 min at 4°C and resuspended in radioimmunoprecipitation assay buffer and protease inhibitor (Sigma-Aldrich; St Louis, MO) on ice for 10 min. The samples were centrifuged, and the protein-containing supernatant was collected. The protein concentrations were assessed using a bicinchoninic acid protein concentration kit (Pierce Protein Research Products; Rockford, IL). Equal concentrations of protein mixed with NuPAGE lithium dodecyl sulfate sample buffer and NuPAGE reducing agent (Invitrogen) were heated at 70°C for 10 min. Samples then were loaded on NuPAGE 4% to 12% Bis(2-hydroxyethyl) aminotris (hydroxymethyl) methane gel (Invitrogen). Protein electrophoresis was carried out at 200 V for 1 h at room temperature. Sample proteins were transferred to a 0.25- μm polyvinylidene fluoride membrane (Invitrogen) in NuPAGE transfer buffer at 4°C (30 V). The membranes were then blocked with 5% nonfat milk in Phosphate Buffer Saline Tween-20 for 1 h at 23°C. The membranes were incubated with primary mouse antihuman monoclonal AKR1B10 antibody (clone 1A6, 2 $\mu\text{g}/\text{mL}$, 1:500 dilution) (Sigma-Aldrich) for 1 h. Detection was performed using secondary horseradish peroxidase-labeled goat antimouse antibody (1:5,000 dilution) (Santa Cruz Biotechnology; Santa Cruz, CA) and an enhanced chemiluminescent reagent system (GE Healthcare; Pittsburgh, PA). To assess the Western blot analyses quantitatively, the film was digitally imaged, maintaining exposure within the linear range of detection. The contrast was inverted, the pixel intensity of each band determined, and the background pixel intensity for a negative area of the film

of identical size subtracted using MetaMorph image analysis software (Universal Imaging; Downingtown, PA). The membrane was subsequently stripped with Restore Western Blot Stripping Buffer (ThermoFisher Scientific; Rockford, IL) and reincubated with horseradish peroxidase-conjugated anti- β -actin antibody (1:5,000) (Sigma-Aldrich) as a control for equal protein concentration.

Localization of AKR1B10 in Large Airway Epithelium of Healthy Smokers

To determine the airway epithelial localization of AKR1B10 expression, biopsy specimens were obtained by flexible bronchoscopy from the large airway epithelia of five healthy nonsmokers and seven healthy smokers using conventional methods. Immunohistochemistry was carried out on paraffin-embedded endobronchial biopsy specimens. Sections were deparaffinized and dehydrated through a series of xylenes and alcohol. To enhance staining, an antigen-retrieval step was carried out by boiling the sections at 100°C for 20 min in citrate buffer solution (Labvision; Fremont, CA) followed by cooling at 23°C for 20 min. Endogenous peroxidase activity was quenched using 0.3% H_2O_2 , and blocking was performed with normal goat serum to reduce background staining. Samples were incubated with the primary mouse antihuman AKR1B10 antibody (clone 1A6, 2 $\mu\text{g}/\text{mL}$, 1:500 dilution) (Sigma-Aldrich) overnight at 4°C. Isotype-matched mouse IgG was used as the control. Vectastain Elite ABC kit (Vector Laboratories; Burlingame, CA) and 3-amino-9-ethylcarbazole substrate kit (Dako; Carpinteria, CA) were used to detect antibody binding, and the sections were counterstained with hematoxylin (Sigma-Aldrich) and mounted using glycerol vinyl alcohol aqueous mounting medium (Zymed; San Francisco, CA). Brightfield microscopy was performed using a Nikon Microphot microscope (Nikon Instruments; Melville, NY), and images were captured with an Olympus DP70 CCD camera (Olympus America; Center Valley, PA).

Airway Epithelial Cells Exposed to CSE

Aqueous CSE was generated from the combustion of one cigarette (Marlboro Red; Phillip Morris USA; Pittsburgh, PA) bubbled through 12.5 mL of culture medium following a slightly modified method described previously.²¹ This medium, defined as 100% CSE, was adjusted to pH 7.4 and filtered through a 0.22- μm filter. Different concentrations of CSE diluted with the culture medium were used, ranging from 0.1% to 20%. Human airway epithelial cell line 16 human bronchial epithelium (16HBE) cells²² were exposed to freshly prepared CSE for 72 h. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche Applied Science; Indianapolis, IN).²³ Viability was expressed as a percentage of the values (corresponding to 100%) of untreated cells. AKR1B10 gene expression was assessed with TaqMan real-time PCR. To further evaluate whether AKR1B10 gene expression was modified by exposure of the cells to CSE, cells already treated with different concentrations of CSE were further cultured for another 7 days with fresh medium (no CSE) changed every other day. Cells were collected, and TaqMan real-time PCR was used to assess AKR1B10 gene expression after CSE removal.

Transfection of AKR1B10 Into Airway Epithelial Cells

To assess whether upregulation of AKR1B10 in airway epithelium enhances the conversion of retinal to retinol, 16HBE cells were plated in 10-cm cell culture plates (BD Biosciences; San Jose, CA). The next day, cells were transfected with an AKR1B10 plasmid driven by the phosphoglycerate kinase promoter, or a control plasmid, using Lipofectamine LTX (Invitrogen) by following the manufacturer's protocol. After 48 h, cells were treated with all-*trans*-retinal (R2500; Sigma) for 4 h, and cells and supernatant were collected.

Table 1—Demographics of the Study Population and Biologic Samples

Parameter	Large Airway		Small Airway	
	Healthy Nonsmokers	Healthy Smokers	Healthy Nonsmokers	Healthy Smokers
No.	21	31	51	58
Male sex, No.	15	21	36	38
Age, y	40.8 ± 7.9	43.5 ± 6.5	41.3 ± 11.4	42.9 ± 7.2
Race				
Black	10	20	24	35
White	7	7	20	14
Other	4	4	7	9
Smoking history, pack-y	0	28.4 ± 18.4	0	27.5 ± 16.7
Urine nicotine, ng/mL	Negative	745 ± 904	Negative	1,231 ± 1672
Urine cotinine, ng/mL	Negative	973 ± 689	Negative	1,182 ± 988
Blood carboxyhemoglobin, % ^a	0.6 ± 0.9	2.0 ± 1.9	0.3 ± 0.7	1.8 ± 1.9
Pulmonary function parameters				
FVC, % predicted	106 ± 13	110 ± 11	102 ± 25	109 ± 13
FEV ₁ , % predicted	107 ± 17	110 ± 12	106 ± 15	107 ± 14
FEV ₁ /FVC, % observed	82 ± 5	81 ± 5	82 ± 6	80 ± 5
Total lung capacity	95 ± 26	103 ± 11	97 ± 24	100 ± 12
DLCO	101 ± 16	95 ± 11	99 ± 14	94 ± 11
Epithelial cells, No. recovered × 10 ⁶	7.6 ± 3.8	7.0 ± 3.3	6.4 ± 2.9	7.2 ± 3.0
Epithelial cells, %	99.7 ± 0.5	99.8 ± 0.5	99.2 ± 1.1	99.1 ± 1.3
Inflammatory cells, %	0.2 ± 0.5	0.2 ± 0.5	0.8 ± 1.1	0.8 ± 1.3
Differential cell count, %				
Ciliated	53.9 ± 6.6	47.8 ± 13.7	73.3 ± 8.2	65.7 ± 12.5
Secretory	10.1 ± 4.5	9.9 ± 4.1	6.7 ± 3.5	9.1 ± 4.5
Basal	22.4 ± 3.5	25.9 ± 9.9	12.0 ± 6.0	12.7 ± 6.7
Undifferentiated	13.8 ± 5.2	16.5 ± 8.9	7.4 ± 3.1	11.9 ± 6.7

Data are presented as mean ± SD, unless otherwise indicated. DLCO = diffusing capacity of the lungs for carbon monoxide.

^aVenous carboxyhemoglobin is a secondary marker of current smoking; in nonsmokers the normal value is < 1.5%.

Retinoids were extracted, and samples were run on high performance liquid chromatography using a reverse-phase column.²⁴ Elution was monitored at 340 nm on a photodiode array detector (Waters Corporation; Milford, MA). The experiment was performed at least three times, starting with freshly plated cells each time.

Statistical Analysis

HG-U133 Plus 2.0 microarrays were analyzed using GeneSpring software (Agilent Technologies). Average expression values in large airway and small airway samples were calculated from normalized expression levels for healthy nonsmokers and healthy smokers. *P* values were obtained using Benjamini-Hochberg correction to limit the false-positive rate. Statistical comparisons between continuous variables were calculated using an unpaired, two-tailed *t* test with unequal variance. Statistical comparisons for categorical data were achieved using χ^2 test. A *P* < .05 was considered significant.

Web Deposition of Data

All data have been deposited in the Gene Expression Omnibus site (<http://www.ncbi.nlm.nih.gov/geo>), which was created by the National Center for Bioinformatics. Accession number is GSE18385.

RESULTS

Study Population

A total of 52 large airway samples from 21 healthy nonsmokers and 31 healthy smokers and 109 small

airway samples from 51 healthy nonsmokers and 58 healthy smokers were analyzed on Affymetrix HG-U133 Plus 2.0 microarrays (Table 1). For the large airway samples, there were no differences between groups with regard to ancestral background (*P* > .4), sex (*P* > .1), and age (*P* > .2). For the small airway samples, there also were no differences between groups with regard to ancestral background (*P* > .2), sex (*P* > .9), and age (*P* > .4). All participants tested HIV negative, and blood and urine parameters within normal ranges (*P* > .05 for all comparisons). Smokers had an average smoking history of 28.4 ± 18.4 pack-years for the large airway samples and 27.5 ± 16.7 pack-years for the small airway samples, with urine nicotine and cotinine and venous blood carboxyhemoglobin levels confirming current smoking status of these individuals. Pulmonary function testing revealed normal lung function in healthy nonsmokers and healthy smokers. The chest radiographs were normal in all participants.

Expression of *AKR1B10* in the Airway Epithelium

Airway epithelial cells were obtained by fiberoptic bronchoscopy and brushing of the large (third- to fourth-order) and small (10th- to 12th-order) airways. The numbers of cells recovered ranged from 2.5 × 10⁶ to 18.2 × 10⁶ (Table 1). In all cases, ≥ 96% of cells recovered were epithelial cells. The various categories

FIGURE 1. Aldo-keto reductase (AKR) 1B10 gene expression levels in large and small airway epithelia from healthy nonsmokers and healthy smokers. **A**, Average normalized gene expression levels of AKR1B10 assessed with microarray HG-U133 Plus 2.0 in the large airway epithelium of 21 healthy nonsmokers and 31 healthy smokers. **B**, Average normalized gene expression levels of AKR1B10 assessed using microarray HG-U133 Plus 2.0 in the small airway epithelium of 51 healthy nonsmokers and 58 healthy smokers. **C**, TaqMan real-time polymerase chain reaction (PCR) confirmation of AKR1B10 gene expression in the large airway epithelium in a subset of 14 healthy nonsmokers and 14 healthy smokers. **D**, TaqMan real-time PCR confirmation of AKR1B10 gene expression in the small airway epithelium in a subset of 18 healthy nonsmokers and 18 healthy smokers.

of airway epithelial cells were as expected from the large and small airways.^{18,19}

Using the HG-U133 Plus 2.0 array and the criteria of Affymetrix Detection Call of Present in $\geq 50\%$ of either healthy nonsmokers or healthy smokers, AKR1B10 was expressed in both large and small airway epithelia. AKR1B10 expression was upregulated significantly in healthy smokers compared with healthy nonsmokers as follows: 21.0-fold in the large airway epithelium ($P < .0001$) (Fig 1A) and 23.1-fold in the small airway epithelium ($P < .0001$) (Fig 1B). For smokers with COPD ($n = 29$), there was also a significant increase in AKR1B10 expression level in the small airway epithelium (31-fold; $P < .00001$), although this change was not significant compared with the level in smokers ($P = .06$). For ex-smokers ($n = 2$), the average gene expression of AKR1B10 in the small airway epithelium was comparable to healthy nonsmokers (not shown), which is consistent with Shah et al,²⁵ who reported on a larger number of ex-smokers whose AKR1B10 expression level was more like that of nonsmokers than smokers.

To confirm the results obtained from microarray studies, TaqMan real-time PCR was carried out on RNA samples from the large airways of 14 healthy nonsmokers and 14 healthy smokers and small airways of 18 healthy nonsmokers and 18 healthy smokers (Figs 1C, 1D). The TaqMan data confirmed the upregulation of AKR1B10 mRNA expression in healthy smokers compared with healthy nonsmokers (large airway, 28.2-fold increase; $P < .02$; small airway, 27.8-fold increase; $P < .01$).

Western blot analysis on both large airway (four healthy nonsmokers and four healthy smokers) and small airway (five healthy nonsmokers and five healthy smokers) samples from the participants whose samples were used for the microarray was used to assess AKR1B10 expression quantitatively (Figs 2A, 2B). Quantitative analysis revealed increased AKR1B10 expression in healthy smokers compared with healthy nonsmokers ($P < .05$) (Figs 2C, 2D). In the large airway epithelia samples, all four smokers showed high expression of AKR1B10. In nonsmokers, only one showed a low level of AKR1B10 expression; AKR1B10 protein could not be detected in other participants. In small airway epithelia, all five smokers showed expression of AKR1B10, and four out of the five showed very high expression. On the contrary, all five healthy nonsmokers did not show obvious bands, indicating the low expression level. We found that both AKR1B10 mRNA and protein levels were highly variable, which is consistent with our previous finding that genetic diversity is likely within a subset of genes with highly variable individual-to-individual responses of the small airway epithelium to smoking.²⁶

Immunohistochemical Assessment of AKR1B10 Expression

To localize AKR1B10 expression in airway epithelial cells, immunohistochemical staining was assessed on endobronchial biopsy specimens from the large airways of healthy nonsmokers and healthy smokers. Positive staining for AKR1B10 was observed in six out of seven healthy smokers but only one out of five healthy nonsmokers (Figs 3A-C). The AKR1B10 signal was mainly detected in the cytoplasm of ciliated cells of airway epithelial cells of healthy smokers. A small portion of basal cells were also positive for AKR1B10. No staining was detected in goblet cells.

Acute Exposure CSE Increases AKR1B10 Gene Expression in Human Airway Epithelial Cells

To assess whether the acute exposure of CSE in human airway epithelial cells affects AKR1B10 gene expression, 16HBE cells were cultured on 12-well plates and exposed to freshly made CSE for 72 h. Cell

FIGURE 2. Western blot analysis of AKR1B10 protein expression in large and small airway epithelia. A, Proteins were extracted from large airway epithelial cells of four healthy nonsmokers and four healthy smokers, and the same gel was probed with anti-β-actin antibody, a control for protein loading (bottom). B, Proteins were extracted from small airway epithelial cells of five healthy nonsmokers and five healthy smokers, with the same gel probed with anti-β-actin antibody for comparison. C, Quantification by densitometry of the ratio of AKR1B10 to β-actin comparing nonsmokers to smokers in large airway epithelium. D, Quantification by densitometry of the ratio of AKR1B10 to β-actin comparing nonsmokers to smokers in small airway epithelium. Error bars represent the SE. See Figure 1 legend for expansion of abbreviation.

viability was analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and AKR1B10 gene expression was analyzed using TaqMan real-time PCR. Decreased cell viability compared with control cells was observed with concentrations of CSE of 20%, 10%, and 5%. On the contrary, the cell viability was 100% in 0.1% CSE and 92% in 1% CSE medium compared with controls (Fig 4A). Based on these data, we chose 0.1% and 1% CSE for the CSE in vitro exposure studies. Compared with controls, AKR1B10 gene expression increased in a dose-dependent pattern, with a 2.1-fold increase in the 0.1% CSE group ($P < .01$) and a 4.6-fold increase in the 1% CSE group ($P < .001$) compared with no-CSE controls (Fig 4B). To investigate further whether AKR1B10 gene expression depended on the presence of CSE, fresh medium without CSE was added to the cells that had been treated with CSE for 3 days. After 7 days, AKR1B10 gene expression was assessed. Interestingly, AKR1B10 expression returned to basal levels similar to that of controls ($P > .05$) (Fig 4B), which is consistent with the conclusion that the upregulated AKR1B10 expression depended on the presence of CSE.

Effect of AKR1B10 Expression on Retinoid Metabolism

When an AKR1B10 plasmid or a control plasmid was transfected into 16HBE cells and the cells then treated with 10 μM all-*trans*-retinal for 4 h, conversion of retinal to retinol was increased in the cells transiently transfected with the AKR1B10 plasmid. Compared with the empty vector control (negative control), AKR1B10 transfected cells showed a higher retinal-to-retinol conversion rate after 4 h of culture in the presence of retinal ($P < .01$) (e-Figure 1).

DISCUSSION

AKR1B10, a member of the AKR family, has been suggested as an early detection marker and treatment target for NSCLC.¹² Using microarray analysis to compare gene expression of the airway epithelium in healthy smokers and healthy nonsmokers, we found AKR1B10 was highly upregulated in both large and small airway epithelia of healthy smokers. The microarray data were confirmed at both the mRNA level (TaqMan real-time PCR) and the protein level

FIGURE 3. Immunohistochemical assessment of large airway epithelium for expression of AKR1B10 in healthy nonsmokers and healthy smokers. A, Isotype mouse IgG control subjects for a healthy nonsmoker and healthy smoker. B, AKR1B10 for healthy nonsmokers. C, AKR1B10 for healthy smokers. Bar = 10 μ m. See Figure 1 legend for expansion of abbreviation.

(Western blot analysis and immunohistochemistry). AKR1B10 was localized to normal human airway epithelial cells, including ciliated and basal cells. Consistent with these data, we found AKR1B10 gene expression could be induced in human airway epithelial cells in a dose-dependent manner in vitro when exposed to CSE and that airway epithelial cells transfected with AKR1B10 had a higher conversion of retinal to retinol. Together, these data demonstrate that AKR1B10 is a smoking-responsive gene, and its upregulation is not limited to squamous metaplasia and cancer tissues but occurs in the airway epithelium in response to smoking.

AKR1B10

AKR1B10 was first cloned from the small intestine and called “aldose reductase-like 1” protein due to its

FIGURE 4. In vitro upregulation of AKR1B10 expression in 16 human bronchial epithelial (16HBE) airway cells induced by exposure to CSE. The 16HBE cells were treated with different concentrations of CSE for 3 days. MTT and AKR1B10 gene expression then were quantified using TaqMan real-time PCR. A, MTT assessment showed the effect of CSE on 16HBE viability. B, Effect of CSE on AKR1B10 expression in 16HBE cells using 0.1% and 1% CSE concentrations that do not affect viability. AKR1B10 gene expression was upregulated when treated with both 0.1% and 1% CSE. Cells treated with 0.1% and 1% CSE for 3 days were changed with fresh medium without CSE and cultured for another 7 days. AKR1B10 gene expression was quantified again using TaqMan real-time PCR. * $P < .01$ compared with no-CSE control subjects. Error bars represent the SD. CSE = cigarette smoke extract; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. See Figure 1 legend for expansion of other abbreviations.

high sequence identity with AKR1B1.¹¹ Similar to other members of the AKR family, AKR1B10 functions as reducer of a variety of carbonyl compounds, such as aliphatic and aromatic aldehydes, ketones, steroids, prostaglandins, sugars, and xenobiotics^{11,27-30} The physiologic function of AKR1B10 is not fully defined, but recent studies have demonstrated that AKR1B10 has high activity with respect to retinal metabolism.¹⁰ AKR1B10 is the most active AKR to modify all-*trans*-retinal, with a catalytic turnover constant/km value about 100-fold higher than other AKRs.^{8,10}

AKR1B10 and Lung Cancer

Based on the knowledge that retinoic acid is an important factor in protecting the airway epithelium

from squamous metaplasia and carcinogenesis, the finding that AKR1B10 expression is upregulated in healthy smokers may reflect one early process in the development of carcinogenesis in smokers. A study assessing gene expression differences between lung cancer tissues and normal lung tissues identified AKR1B10 as upregulated in smokers with squamous cell carcinoma but not in smokers with adenocarcinoma.³¹ In another study, AKR1B10 was expressed in most smokers with squamous cell carcinoma, in some smokers with adenocarcinoma, and occasionally in smokers with squamous metaplasia.¹² AKR1B10 also was identified in squamous metaplasia associated with honeycomb lesions of smokers with idiopathic pulmonary fibrosis (also referred to as usual interstitial pneumonia), a disease that carries an increased risk of lung cancer in smokers.³²

Theoretically, AKR1B10 might promote carcinogenesis by promoting cell proliferation, promoting cell survival, or inhibiting the conversion of all-*trans*-retinal to retinoic acid. The first mechanism is supported by evidence of the positive correlation of AKR1B10 expression and putative poor prognosis factors, such as high Ki-67 and high cyclin E in NSCLC.¹² The second is supported by the evidence that knockdown of AKR1B10 in human colon and lung carcinoma cells result in caspase-3-mediated apoptosis.^{33,34} The third is supported by in vitro and in vivo studies demonstrating the activity of AKR1B10 in retinoic acid metabolism.^{8,10} Because retinoic acid is the vital factor in human airway epithelial differentiation, a local deficiency of retinoic acid promotes squamous metaplasia and carcinogenesis of the airway epithelium.^{14,15} The data demonstrating that overexpression of AKR1B10 resulted in preferential conversion of exogenous retinal to retinol are consistent with this third mechanism.

AKR1B10 as a Marker for Lung Cancer

AKR1B10 has been considered a specific early lung cancer marker because of its high levels of expression in lung cancer tissue and squamous metaplasia.¹² The data in the present study suggest that AKR1B10 is highly and widely induced in healthy smokers with no evidence of lung cancer. These results raise the following question: Is AKR1B10 a specific lung cancer early detection marker, a smoking-induced protein relevant to the very early stage of carcinogenesis prior to metaplasia, or just a bystander? The data show that the upregulation of AKR1B10 in healthy smokers is prominent and widespread, with AKR1B10 expression in the basal cell population as well as in the differentiated airway epithelial cell types, such as ciliated cells. Consistent with the in vivo data, the in vitro experiments demonstrate that acute exposure of CSE to human

airway epithelial cells is enough to increase AKR1B10 gene expression in a dose-dependent pattern, consistent with the upregulation of AKR1B10 gene expression in normal epidermal keratinocytes and an oral carcinoma cell line after in vitro treatment with CSE.²³ In the context of these observations, AKR1B10 is not a marker of cancer per se. However, its upregulation and function in detoxification and the retinoic acid pathway suggest that it may play an early role in carcinogenesis.

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Dr G. Wang: performed experimental work, including Western analysis.

Ms Ricard: contributed to the retinoic acid experiments.

Ms Ferris: performed experimental work related to immunohistochemistry.

Ms Strulovici-Barel: contributed to compilation and analysis of microarray data.

Ms Salit: contributed to quality control and statistical analyses of microarray data.

Dr Hackett: contributed to experiment design, manuscript preparation, and revision.

Dr Gudas: contributed to experiment design and critical interpretations related to retinal metabolism.

Dr Crystal: contributed to the overall concept, experiment design, interpretation, and the scientific context of the work performed.

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