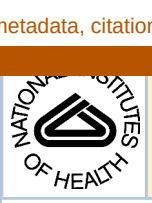

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INCREASED NASAL EPITHELIAL CILIARY BEAT FREQUENCY ASSOCIATED WITH LIFESTYLE TOBACCO SMOKE EXPOSURE

Haibo Zhou[1,3], **Xiaoyan Wang**[1,3], **Luisa Brighton**[3], **Milan Hazucha**[3], **Ilona Jaspers**[2,3], and **Johnny L. Carson**[2,3]

[1] Department of Biostatistics, The University of North Carolina at Chapel Hill

[2] Department of Pediatrics, The University of North Carolina at Chapel Hill

[3] Center for Environmental Medicine, Asthma, and Lung Biology, The University of North Carolina at Chapel Hill

Abstract

The ciliated epithelium of the respiratory airways is one of the first vital systemic surfaces in contact with the ambient air. *Ex vivo* nasal epithelial ciliary beat frequency (CBF) at room temperature is on the order of 7–8 Hz but may be stimulated by irritant exposure. The upregulation of CBF in response to acute irritant exposure is generally considered to be a transient event with eventual return to baseline. However, studies of CBF dynamics in response to typical lifestyle exposures are limited. This study assessed nasal epithelial CBF among human subjects as a function of quantifiable lifestyle tobacco smoke exposure. Nasal epithelial biopsies were obtained from human subjects with well documented histories of tobacco smoke exposure. CBF was determined using a digital photometric technique and concurrent assays of nasal nitric oxide and urine cotinine and creatinine were performed. Mean CBF among active smokers and non-smokers exposed to environmental tobacco smoke (ETS) was elevated over non-smokers. Although there were dramatic differences in relative levels of tobacco smoke exposure, CBF values among tobacco smoke-exposed groups were comparable. Parallel *in vitro* studies of cultured nasal epithelium exposed to cigarette smoke condensate further supported these observations. These studies suggest that persistent elevation in nasal epithelial CBF is an early, subtle, physiologic effect associated with lifestyle tobacco smoke exposure. The molecular mechanisms that upregulate CBF may also create a cell molecular milieu capable of provoking the eventual emergence of more overt adverse health effects and the pathogenesis of chronic airway disease.

Introduction

Ciliated cells lining the conducting airways are integral elements of mucociliary clearance that serve to transport secretions and potentially injurious inspired materials out of the airways. Ciliary activity is hypothesized to respond to acute challenge by transiently upregulating ciliary beat frequency (CBF) through several mechanisms (1–3). Although critical elements of this primary respiratory defense mechanism, ciliated cells are themselves highly vulnerable to structure/function changes imposed by infectious agents (4) and their products (5), irritants (6,7), and inflammatory processes (8). Persistent challenges resulting in injury and death of ciliated cells may lead to remodeling of the epithelial layer with the remodeled layer exhibiting a reduced complement of ciliated cells accompanied by an increase in secretory cells, excessive

Corresponding Author and Address for Reprints: Johnny L. Carson, Ph.D., Center for Environmental Medicine, Asthma, and Lung Biology, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7310, Phone: 919-966-0763, Fax: 919-966-9863, E-mail: E-mail: jcarson@med.unc.edu (publishable).

mucus secretion (9), and diversion of optimal mucociliary function in ways that may lead to or exacerbate a variety of disease processes including asthma, chronic obstructive pulmonary disease, chronic bronchitis, and neoplasia (10).

The ability of the epithelial lining of the respiratory airways to respond to acute challenge from potentially injurious inhaled agents by upregulating ciliary beat frequency and secretory processes to protect and clear the airways is well documented. The transition of healthy airway epithelium to an inflamed, hyperplastic, dysfunctional epithelial layer and the emergence of disease processes due to chronic exposure to irritants such as tobacco smoke also are well documented. However, subtle physiologic changes in ciliated cell function in response to persistent irritant challenges that may portend later more overt adverse health effects have not been critically examined particularly as they relate to lifestyle exposures. In the present study, we assessed CBF in nasal epithelial biopsies obtained from human subjects reporting a broad spectrum of lifestyle tobacco smoke exposures ranging from active smokers to individuals exposed to environmental tobacco smoke (ETS) in occupational and/or domestic environments. Beat frequency dynamics in these studies were compared to responses of well differentiated cultured human nasal epithelium from non-smoking subjects to *in vitro* cigarette smoke condensate (CSC) exposure.

Methods

Human subjects

This study was reviewed and approved by The University of North Carolina at Chapel Hill Office of Human Research Ethics (IRB). A total of 61 subjects with a broad range of smoking histories were recruited. All the subjects, regardless of smoking history, reported subjectively that their overall health was good. There were neither asthmatics nor participants with other previously diagnosed chronic respiratory diseases. Participants self-reported their status as belonging to one of three groups: 1) non-smoking (NS), 2) smoking (S), or 3) non-smoking but domestically or occupationally exposed to environmental tobacco smoke (NS/ETS). Individuals identifying themselves as smokers subjectively reported recent cigarette use as no more than 2 packs of cigarettes per day. There were 21 non-smokers, 27 smokers, and 13 ETS-exposed individuals in the study groups. There were 29 males and 32 females and race distribution was consistent with population demographics of central North Carolina. The median age was 26 years for non-smokers, 35 years for smokers, and 26 years for ETS-exposed subjects.

Nasal biopsy and determination of ciliary beat frequency

A non-invasive nasal biopsy to retrieve ciliated epithelium lining the inferior surface of the inferior nasal turbinates was performed on each subject for the determination of CBF. All subjects were sampled at random times during a typical work day. No efforts were made to specifically target epithelial sampling proximal to times when smoking subjects had recently smoked or times when NS/ETS subjects had encountered lifestyle ETS exposures. Nasal biopsies were obtained without anesthesia using a sterile, plastic nasal curette (Rhino-probe, Arlington Scientific, Arlington, TX). Immediately upon acquisition, small fragments of the mucosa were transferred to a microscope slide and cover slipped. Each specimen was viewed at room temperature (25C) under a Nikon Diaphot inverted microscope equipped with a 40x phase contrast objective. For determination of ciliary beat frequency (CBF), the microscope was interfaced to a high speed digital camera (Basler AG, Ahrensburg, Germany) and a computer running software designed for making CBF measurements (11) (Ammons Engineering, Mt. Morris, MI). Mean point CBF was determined at 50 points for each specimen by assessing five random points in each of ten random microscopic fields.

Cotinine assays

Each study subject provided a urine specimen coordinate with the time of the nasal biopsy to obtain a quantitative measure of tobacco smoke exposure. Cotinine analyses were performed using a commercially available ELISA test (Bio-Quant, San Diego, CA).

Statistical analyses

The observed data structure yielded 50 CBF point observations per individual. To optimize the information provided by the repeated CBF point data a mixed model approach to detect differences in CBF among the test groups was used. A mixed linear regression model with an individual-specific random intercept modeled the effect of smoking status as well as adjusting for the subject's age, race, and gender. The random intercept allowed adjustment for the potential correlation of the repeated measurements among individuals. All analyses were carried out using SAS version 9.1 (SAS Institute Inc., Cary, NC). We applied the mixed model approach to both the full data set, where the three smoking status groups were compared simultaneously and to the subsets (NS vs. NS/ETS-exposed and NS vs. S), respectively. The main conclusions from the full data set analysis agreed with those from the subset analysis.

In vitro study of cultured ciliated nasal epithelium exposed to cigarette smoke condensate (CSC)

Nasal biopsies from five non-smoking subjects were used to establish air-liquid interface (ALI) epithelial cultures for a comparative *in vitro* assessment of ciliary function in response to exposure to cigarette smoke condensate. The biopsies were initially expanded mitotically using conventional tissue culture techniques and subsequently seeded onto Vitrogen coated transwells for establishment of ALI cultures. Well differentiated cultures appeared in approximately one month and these were subjected to 250 μl of vehicle or CSC in concentrations of 1, 10, and 100 μg/ml (12). CBF was determined at 5 points in each of 10 random fields. Cells from one subject were incubated in the presence of DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate)(Invitrogen, Eugene, OR), a fluorescent marker of nitric oxide production, with exposure to 100 μg/ml CSC or vehicle and examined after one hour by episcopic fluorescence microscopy for enhanced fluorescence.

Results

Comparative CBF characteristics of epithelial biopsies from non-smoking and smoke-exposed subjects

Baseline mean CBF in nasal biopsies from well documented non-smokers at room temperature (25C) was 7.28±0.32 Hz. This contrasts with an increase in mean CBF to 7.99±0.35 Hz among NS/ETS subjects, and 8.17±0.44 Hz among light smokers, and 8.54±0.48 among heavy smokers respectively. The mean CBF among subjects exposed to tobacco smoke by any route was 10–17% greater than for non-smokers. Although the median cotinine level of the NS/ETS group was only slightly above that of non-smokers, mean CBF among subjects in this group was notably higher than that observed in well documented non-smokers. The pattern of increasing CBF associated with increasing smoke exposure persisted across assessments of minimum, median, and maximum CBF as well (Table 1). These observations illustrate the transition of CBF characteristics among NS/ETS individuals toward frequencies more characteristic of smokers. They also suggest that non-smokers experiencing lifestyle exposures to ETS may exhibit persisting increases in CBF with relatively low levels of exposure. Figure 1 depicts the relationship between mean CBF per individual and the $\log_{10}$ ([cotinine]/[creatinine]). The line in the graph is the fitted regression line using the mixed model approach with the fitted slope=0.016, SE=0.07, p=0.027. This plot not only illustrates the increased CBF

of tobacco smoke-exposed subjects but also the clear demarcation of CBF characteristics of non-smokers and smoke-exposed individuals regardless of the means of exposure.

Using the mixed model approach with adjustment for subject age, race, and gender the full data set analysis, and using non-smokers as the reference group, the mean CBF in the NS/ETS and the smoker groups was significantly higher than the non-smoker group, with p=0.057 and p=0.033 respectively. The estimated coefficients and their estimated standard errors for the NS/ETS and the smoker groups are 1.04 Hz (SE=0.54) and 1.01 Hz (SE=0.47), respectively. The baseline mean CBF of non-smokers is estimated at 7.56 Hz (SE=0.74). There was no detectable difference in CBF between the NS/ETS and smoker groups. These results were validated by a subset analysis using the same mixed model approach comparing the non-smoker group vs. the NS/ETS and non-smoker (NS) group vs. the smoker (S) group respectively. It is clear that both the NS/ETS and smoker (S) groups have elevated CBF compared with the NS group. In fact, the data suggest that the NS/ETS group does not represent an intermediate group between non-smokers and smokers, but rather exhibit CBF characteristics that are more consistent with those of smokers.

Relative levels of tobacco smoke exposure based on urine cotinine levels

Cotinine assays of non-smokers documented low background levels of tobacco smoke exposure. However assays of smokers demonstrated great variability suggesting that, despite histories of no more than 2 pack/day, tobacco use among smokers ranged from casual, intermittent use to heavy regular use. We therefore divided smokers into light smokers and heavy smokers based on the median cotinine level of all smokers as a dividing line separating these two sub-groups. The CBF characteristics and the [cotinine]/[creatinine] ratios of the four sub-groups are presented in Table 1.

Nasal nitric oxide (nNO) determinations as a function of tobacco smoke exposure status

Nasal nitric oxide determinations performed immediately prior to nasal biopsy showed no significant differences in nNO concentration between non-smoking and tobacco smoke-exposed subject groups (Table 1). However, it is noteworthy that among the tobacco-smoke exposed groups that the highest levels of mean nNO were observed among heavy smokers. Also, nNO concentrations exhibited greater variability among subjects in all the tobacco smoke-exposed groups than in the non-smoker group.

Correlative in vitro studies of CBF dynamics in cultured airway epithelium exposed to cigarette smoke condensate

Well differentiated air-liquid interface epithelial cultures derived from biopsies of well documented non-smokers exposed to 1 and 10 μg/ml of CSC did not show a significant increase in CBF relative to assessments of control cells. In contrast, exposure of well differentiated cells to 100 μg/ml CSC produced a significant increase in CBF from 10.61 Hz in controls to 12.03 Hz immediately upon application of CSC to the culture surface. Interestingly, this increase of approximately 13% in CBF was comparable to the increase in CBF seen in fresh biopsies from tobacco smoke-exposed subjects. Control cells incubated in the presence of DAF-FM diacetate exhibited only low background green fluorescence whereas cells exposed to 100 μg/ml CSC fluoresced brightly reflective of the generation of nitric oxide in response to CSC exposure.

Discussion

Previous human and animal experimental studies have reported varying effects of tobacco smoke exposure on mucosal ciliary activity (13–16). Several factors explain these differences including: a) variability in chemical composition and delivery of tobacco smoke, b) severity of exposure or smoking history, c) differences in airway anatomy among species, and d)

methods for quantifying exposure. Our assessment of normal baseline CBF among human subjects is consistent with other reports in the literature. These translational investigations sought to determine whether differences in CBF of human nasal epithelium sampled under lifestyle conditions correlated to quantifiable tobacco smoke exposure. Sampling was not targeted to specific smoking or smoke exposure events thus capturing a broad perspective of CBF associated with lifestyle exposures. We have documented a subtle but statistically significant increase in the mean CBF of smokers and non-smokers exposed to ETS relative to non-smokers and a marked difference in the maximum CBF in tobacco smoke-exposed populations relative to the smoke-free population. Moreover, although the NS/ETS population exhibited levels of urine cotinine that were significantly less than smokers and only slightly higher than non-smokers, increases in CBF in this group were comparable to those of heavy smokers.

Mucociliary clearance efficiently limits and clears irritant gases and particulates from the respiratory airways. The mucociliary transport rate is a function of the viscoelastic properties of mucus and CBF. Numerous studies of chronic smokers have reported decreased bronchial and nasal mucociliary clearance when compared to healthy individuals (17). This likely is due to inflammation of the airways, increased production of mucus particularly of more viscous mucins, and decreased CBF possibly coupled with morphologic changes of ciliated cells and cilia. Our study cohort of active smokers was asymptomatic for respiratory symptoms, reported that their overall health was good, and none had been diagnosed with a chronic respiratory disease. Smokers who have not yet developed respiratory symptoms have been reported to produce more watery mucus and an increase in mucociliary clearance (18). Most likely the increase in clearance was due to increased CBF. Similarly, a study of exposure of healthy individuals to ETS or sidestream (SS) smoke showed an increase in nasal mucociliary clearance and implicitly increased CBF in most of the subjects (19). This contention is further supported by animal studies. High concentrations of many common irritants are typically thought to be ciliotoxic. Indeed, previous *in vitro* studies in this laboratory (unpublished data) have demonstrated a dramatic suppression of CBF by high concentrations of cigarette smoke condensate which is rapidly reversible by washing the cultured cells with fresh medium. However, doses more consistent with exposure levels that might be typically encountered in a smoking environment promote the protective acceleration of CBF. Mucociliary clearance in the rabbit maxillary sinus was accelerated by cigarette smoke exposure (20,21), the increased clearance involving both stimulation of sensory C-fibres and ganglion nicotinic receptors by nicotine, since nicotine-free cigarettes did not affect mucociliary activity. Although changes in physico-chemical properties of mucus may influence CBF, the observed acceleration of clearance in these animals must be due to increased CBF via neural mechanisms. The autonomic control of CBF has been well documented. Stimulation of nicotinic receptors of C-fibres by capsaicin (22) or other agonists (23) also is known to increase tracheal and nasal CBF. Since sampling was not targeted to times proximal to exposure in the present study, these data point to a persistent upregulation of CBF in populations exposed to tobacco smoke whether through active or passive exposure.

While the coordinated interaction of secretion and ciliary activity are obvious, other constitutive and signaling functions optimize mucociliary activity during episodic irritant challenge. There is considerable evidence for the influence of nitric oxide (NO) as a signaling molecule in the upregulation of epithelial CBF (24). This upregulation is protective and typically thought to return to baseline upon cessation of an irritant challenge. Consistent with the findings of the present study, Wyatt et al. (25) have shown that experimental exposure of bovine ciliated cells to hog barn dust increases baseline CBF through a nitric oxide mediated pathway. In the present study, we also demonstrate the generation of nitric oxide and a concomitant increase in CBF in epithelial cells cultured from non-smokers exposed to acutely to CSC *in vitro*. Many chemical components in the tobacco smoke milieu may activate nitric

oxide synthases leading to the generation of nitric oxide and the persistence of CBF acceleration. Although baseline CBF does not appear to be modulated by NO, a variety of agents (1,26–29) have been shown to upregulate ciliary beat frequency through a nitric oxide-cyclic guanosine monophosphate pathway (30). Indeed, endothelial NOS (NOSIII) localizes proximal to ciliary basal bodies (31). Increases in the intracellular calcium flux also have been posed as a mechanism for the upregulation of ciliary beat frequency (3,32) and calcium transients generated by mechanical forces on the epithelium may play into the regulation of ciliary activity through the NO pathway as well (33).

Although upregulation of CBF through a nitric oxide pathway is a plausible mechanism for the results observed in this study, mean nNO concentrations were not significantly different between non-smoking and tobacco smoke-exposed subjects. However, variation in nNO between non-smokers and all smokers or heavy smokers was significant. This observation is likely a reflection of variability in individual smoking habits as well as the interval of time between recent tobacco smoke exposure events, the timing of nNO assays, and the mechanisms whereby nitric oxide is generated and metabolized. While one might hypothesize that irritant based increases in inflammation might lead to increased nNO, other studies (34) report a reduction in production and concentration of NO in the conducting airways. This decline may be associated with oxidant injury from the chemical milieu contained in tobacco smoke. Moreover, the persistent upregulation of CBF through an NO pathway may create a rich background for nitration of critical proteins with potentially pathogenic consequences (35). These studies document the immediate physiologic response of the airway epithelium to upregulate CBF upon exposure to tobacco smoke but more importantly suggest that that protracted exposures promote the persistence of this upregulation. In view of the pro-inflammatory nature of nitric oxide in mediating this event, this persistence may be contributory to the emergence of adverse health effects in the longer term. For example, 3-nitrotyrosine (NO2Tyr) deriving from NO production may promote post-translational modification of alpha-tubulin and thereby alter epithelial microtubules and their attendant functions. Previous studies from this laboratory (36) have shown co-localization of NO2Tyr with epithelial microtubules and the consequent inhibition of intracellular signaling during experimental infection by respiratory syncytial virus.

In summary, these studies suggest that persistent upregulation of CBF represents one of the earliest detectable direct physiologic effects associated with tobacco smoke exposure. While upregulation of CBF in the presence of an irritant is a protective, transient event, extended exposures driven by proinflammatory mechanisms and promoting persistent upregulation of CBF may facilitate the development of a cell molecular milieu predisposed to the emergence of adverse health effects and chronic respiratory disease.

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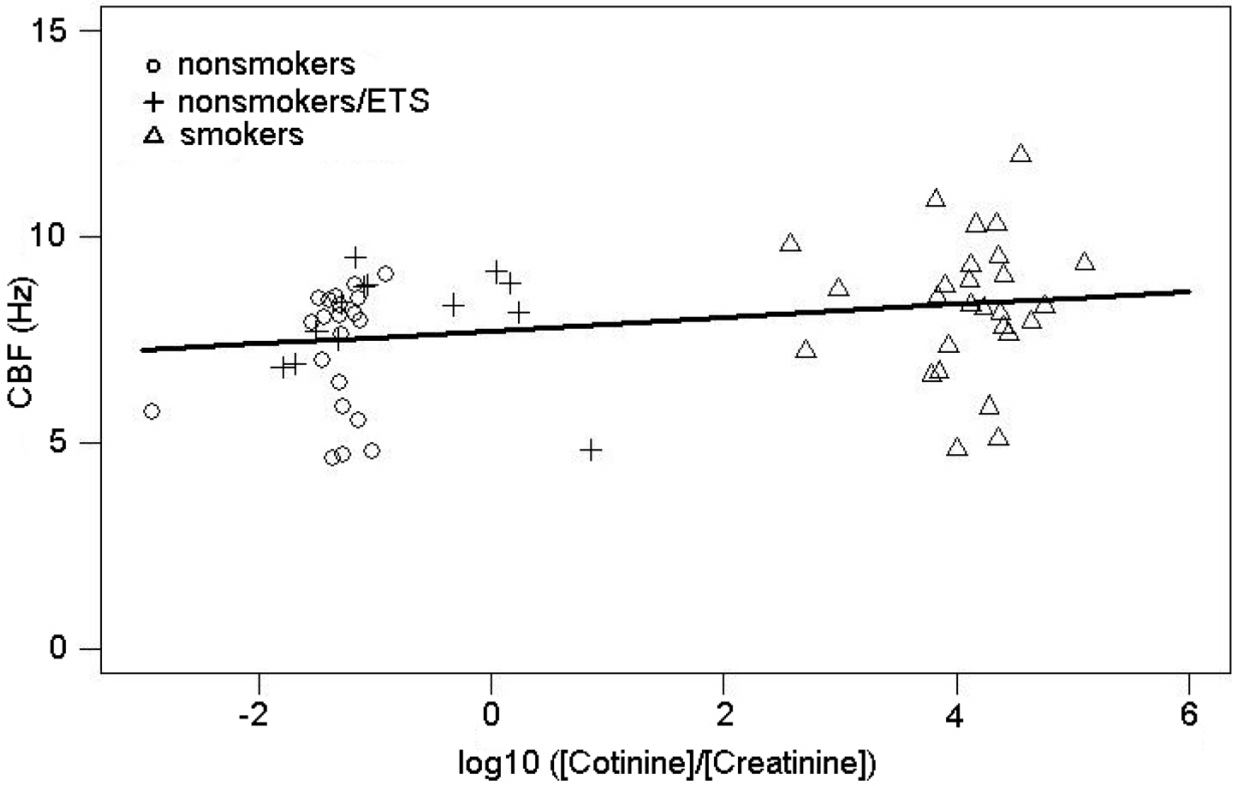

Figure 1.
Scatter plot and regression line of nasal epithelial CBF as a function of cotinine:creatinine ratio among non-smokers, non-smokers exposed to ETS, and active smokers. CBF values are presented as the mean of fifty CBF determinations for each subject.

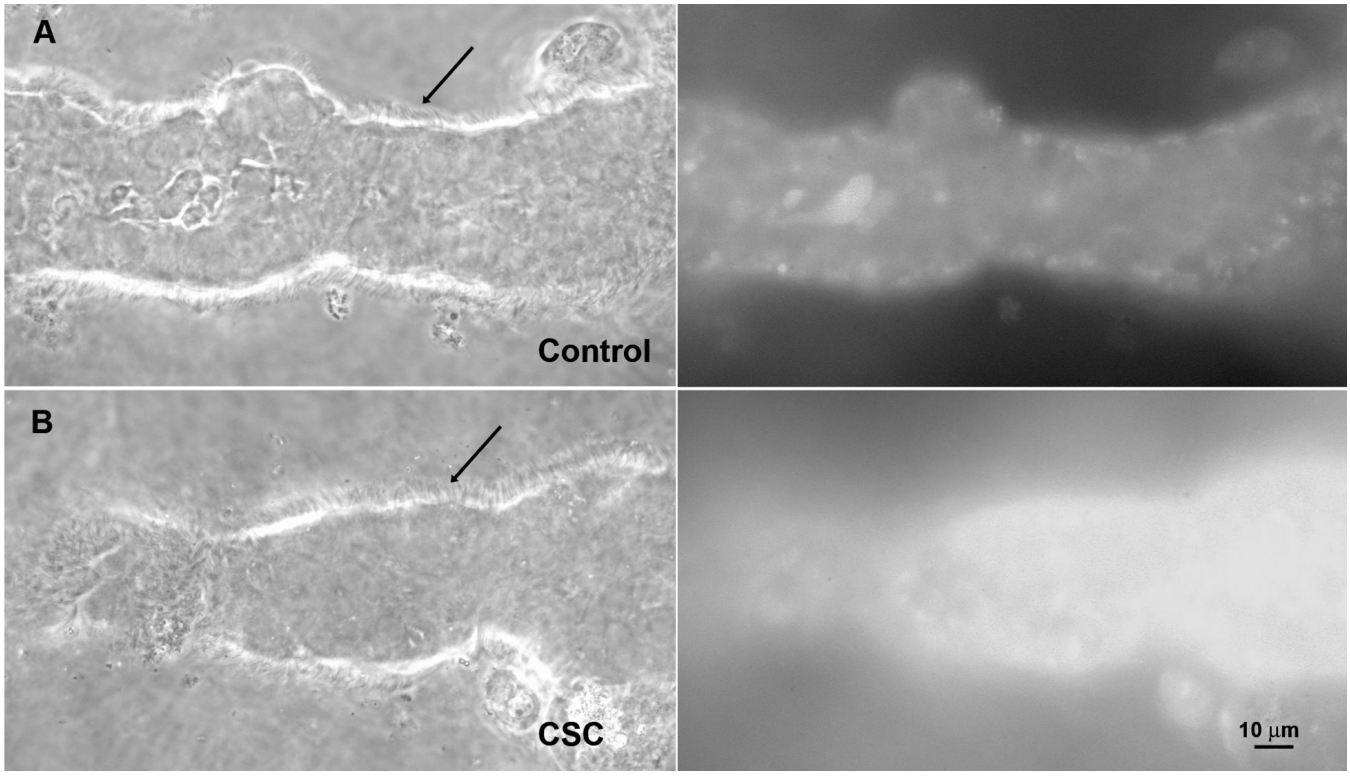

Figure 2.
Comparative fluorescence images of air-liquid interface cultures of nasal epithelium from non-smoking human subjects incubated in the presence of DAF-FM diacetate with and without exposure to 100 μg/ml CSC. Left panels are phase contrast images of cultures showing ciliary beds (arrows). Right panels illustrate increased intensity of fluorescence reflective of generation of nitric oxide in CSC-exposed cells relative to low background fluorescence in control cells.

Table 1

Assays of CBF, urine cotinine, and nasal nitric oxide as a function of tobacco smoke exposure history

Category*	n=	Mean CBF	Standard Error	Min CBF	Median CBF	Max CBF	Mean Log10 ([Cotinine]/[Creatinine)	Nasal Nitric Oxide nl/min Mean ± SD
Non-Smokers	21	7.28	0.32	4.61	7.95	9.08	−1.35	309±80
Non-Smokers/ETS-exposed	13	7.99	0.35	4.84	8.35	9.51	−0.77	317±92
Light Smokers	13	8.17	0.44	4.85	8.57	10.89	3.67	265±104
Heavy Smokers	14	8.54	0.48	5.09	8.28	11.97	4.46	337±168