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Smoke Exposure Exacerbates an Ethanol-Induced Defect in Mucociliary Clearance of *Streptococcus pneumoniae*

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Abstract

Background—Alcoholics and smokers are particularly susceptible to pulmonary infections caused by *Streptococcus pneumoniae*, the pneumococcus. Infection begins when pneumococci colonizing the nasopharynx are aspirated into the lower respiratory tract. The major host defense against this movement is the mucociliary clearance apparatus. Both cigarette smoke and ethanol (EtOH) exposure alter ciliary beating and protein kinase activity in the respiratory mucosa *in vitro*, but their effects on bacterial clearance in the intact animal have not been determined.

Methods—Male Sprague-Dawley rats were exposed twice daily for 12 weeks to either the smoke generated from 30 cigarettes (smoke-exposed) or room air (sham-exposed). For the last five weeks of smoke exposure, the rats were fed Lieber-DeCarli liquid diets containing 0%, 16%, 26% or 36% EtOH calories. The rats then were infected intranasally with *S. pneumoniae*, and movement of the organisms into the lower respiratory tract was quantified by plate counts of the trachea and lungs four hours later. Ciliary beat frequency (CBF) analysis was performed on tracheal ring explants from each animal before and after stimulation with the β -agonist isoproterenol, and tracheal epithelial cell protein kinase C (PKC) activity was measured.

Results—Ingestion of any of the EtOH-containing diets resulted in a dose-dependent increase in movement of *S. pneumoniae* into the rats' lungs. This EtOH-induced defect was augmented further by concurrent smoke exposure, although smoke exposure alone had little effect on *S. pneumoniae* movement. Smoke, but not EtOH exposure, activated tracheal epithelial cell PKC. Increased movement of organisms into lungs correlated with a decrease in CBF, and loss of the ciliary response to isoproterenol.

Conclusion—EtOH ingestion in our model facilitates movement of *S. pneumoniae* into rats' lungs, a phenomenon exacerbated by concurrent smoke exposure. Furthermore, the organism's movement into the lungs correlated with a blunting of the rats' ciliary response to an established stimulus. Defects in mucociliary clearance thus may be one cause of the increased risk of pneumococcal infections in people who abuse alcohol, particularly if they also smoke.

Keywords

Ethanol; Smoking; *Streptococcus pneumoniae*; Cilia

Excessive ethanol (EtOH) ingestion has long been associated with an increased risk of lower respiratory tract infections, including the most common bacterial cause of community-acquired pneumonia, *Streptococcus pneumoniae* (the pneumococcus). Pneumococcal infections in alcoholics often are severe and more likely to result in bacteremia, thereby increasing the

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mortality rate (Ruiz et al., 1999; Young et al. 1989). The effects of alcoholism on the immune system have been carefully examined through both animal models and human studies (as reviewed in Szabo 1999; and Zhang et al., 2002). Little research has been done, however, to elucidate the concurrent effects of smoke exposure and excessive EtOH ingestion, even though greater than 80% of human alcoholics also smoke cigarettes (Bien and Burge, 1990) and smoking alone is a significant risk factor for the development of invasive pneumococcal disease (Aronson et al., 1982; Nuorti et al., 2000). Because smoking and excessive alcohol ingestion are closely linked and both behaviors increase the risk of severe pneumococcal disease, it is important that their concurrent effects on the host are examined thoroughly.

The first step in establishment of pneumococcal pneumonia is colonization of the bacterium in the nasopharyngeal region. The pneumococcus is obviously proficient at colonizing, as about 40% of adult Americans carry the organism during the winter months (Austrian, 1986). To produce a pulmonary pneumococcal infection, however, the organism must travel from the nasopharynx into the lower respiratory tract. To defend itself against such an attack, the host presents an elaborate mechanical defense system known as the mucociliary clearance apparatus to prevent bacteria residing in the nasopharynx from reaching the lung. This defense system is composed of ciliated columnar epithelial cells whose cilia beat in a coordinated manner to move mucous and trapped microorganisms back to the upper respiratory tract. The rate at which bacteria are removed from the airways depends in part on the pace at which the cilia beat, known as ciliary beat frequency (CBF).

CBF is regulated primarily by protein kinase activity, such that it is increased by the activation of protein kinase A (PKA) and protein kinase G (PKG), and decreased by activation of protein kinase C (PKC) (Levin et al., 1997; Salathe et al., 1993; Wong et al., 1998; Wyatt et al., 1998). Using an *in vitro* model, acute EtOH exposure has been shown to activate PKA activity and increase CBF in bovine bronchial epithelium (Sisson et al., 1999). In contrast, chronic exposure of bovine bronchial epithelial cells to EtOH results in a return to original baseline values (Wyatt and Sisson, 2001). *In vitro* exposure of cultured bovine tracheal cells to cigarette smoke extract also has been shown to activate PKC and thus downregulate CBF (Kensler and Battista, 1963; Sisson and Tuma, 1994, Wyatt et al., 2000). Although these separate effects of cigarette smoke or EtOH exposure on CBF have been examined *in vitro*, to our knowledge no *in vivo* studies have been published to date looking at the concurrent effect of the two treatments on PKC activity and CBF, particularly as they relate to bacterial movement from the nasopharynx into the lungs.

We postulated that a defect or alteration in CBF due to smoke exposure and ethanol ingestion would decrease the mucociliary clearance of *S. pneumoniae*, preventing the cilia from effectively keeping the organisms away from the lung. To test this hypothesis we utilized an *in vivo* model of concurrent smoke exposure and ethanol ingestion developed in our laboratory (Gentry-Nielsen et al., 2004). Through the use of computerized frequency analysis of CBF and direct PKC activity assays, we have shown that smoke exposure exacerbates an alcohol-induced defect in ciliary response that correlates with increased movement of organisms into the lungs of rats. We suggest this as one potential cause of the increased risk of pneumococcal pneumonia in the smoking, alcoholic population.

METHODS

Animals

Male, Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) were obtained at 4–5 weeks of age (100–120g) and rested for one week before their first smoke exposure. The rats then were smoke-exposed as described recently by Gentry-Nielsen et al. (2004). Briefly, rats were placed twice daily (once on weekends) for one hour in a whole body smoking chamber

(Teague Enterprises, Davis, CA) through which the smoke generated from 30 standard 2R4F reference cigarettes was pumped (Tobacco and Health Research Institute, University of Kentucky). Sham-exposed rats were placed in similar chambers for equivalent time periods, but were exposed to room air only.

After 7 weeks of smoke or sham exposure, the rats were moved to individual cages and switched from their regular chow diets to the Lieber-DeCarli 35% fat liquid control diet (Dyets, Inc., Bethlehem, PA). After a 3-day acclimation period to the 0% EtOH diet, the rats in each exposure group were placed in four different feeding groups, such that they consumed diets containing 36%, 26%, 16% or 0% ethanol calories. The rats then were concurrently smoke-exposed and EtOH-fed for an additional five weeks before being used in experiments.

Blood alcohol levels were measured using a commercial kit (Sigma Diagnostics, St. Louis, MO) at the end of week 1 and week 3 of concurrent smoke exposure and EtOH ingestion. Blood (200 μ L) for blood alcohol levels was collected by aseptic puncture of the rats' foot veins (Snitily et al., 1991) early in the morning before the a.m. smoke exposure.

Bacteria

S. pneumoniae type 3 (ATCC 6303, American Type Culture Collection, Rockville, MD) was utilized in all experiments. The organism was grown to mid-logarithmic phase in a 5% CO₂ atmosphere in Todd-Hewitt broth supplemented with 5% heat-inactivated rabbit serum. The bacteria then were collected by centrifugation, washed, and resuspended in phosphate-buffered saline (PBS) to a concentration of 1×10^9 colony forming units (cfu)/ml.

Intranasal Infection

Immediately after their morning smoke exposure, the rats were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, New Jersey) and infected intranasally with 1×10^8 cfu of *S. pneumoniae* in 100 μ L of PBS. The rats were held in a vertical position for one minute to allow for aspiration of the fluid.

Cultures of Trachea and Lungs

Four hours after the infection procedure, the rats were euthanized by an intraperitoneal injection of 75mg/kg body weight of pentobarbital (Nembutal, Abbott Laboratories, Abbott Park, Illinois). Immediately after euthanasia, the trachea and lungs were removed aseptically *en bloc* and placed in a sterile petri dish. The trachea was cut off with sterile scissors, and the numbers of pneumococci present were quantified by insertion of miniature cotton swabs (Fisher Scientific, Pittsburgh, PA) into both the distal and proximal ends. The swabs were swirled in separate tubes containing 100 μ L sterile PBS, and serial dilutions of the original suspension were spread onto blood agar plates. The lungs were minced and placed in a sterile tissue grinder and the total volume was adjusted to 10ml with PBS. The tissues then were homogenized and quantitative plate counts were performed on the resulting homogenates.

Ciliary Beat Frequency Measurement

Tracheal ring explants 1–2mm thick were prepared from the trachea and examined by phase contrast microscopy as described previously (Wyatt et al., 2004). Briefly, actively beating ciliated cells were maintained at $24 \pm 0.5^\circ\text{C}$ by a thermostatically controlled heated stage and digitally analyzed using the Sisson-Ammons Video Analysis (SAVA) system (Sisson et al., 2003). Whole field analysis (WFA) was performed using software (Ammons Engineering, Mt. Morris, MI) that automatically analyzed the entire captured image of all ciliated cells in a given field. All cilia amplitudes and frequencies were collated, mapped to the screen image and statistically analyzed to determine the frequency average, standard deviation and standard error

of the entire image. The predominant frequency of all motile cilia was sampled in at least 6 separate fields. All frequencies are expressed as mean $\pm$ SEM from six separate fields. Baseline CBF was recorded in multiple tracheal rings for each rat treatment condition as described (Wyatt et al., 2004). CBF measurements were made both at baseline (immediately after tracheal harvest) and 30 minutes after stimulation *in vitro* with 100 μ M isoproterenol, a known β -agonist that stimulates ciliary beating.

Protein Kinase C Activity Assay

Tracheas from rats in each exposure group were harvested, longitudinally cut, and washed in PBS. Tracheal epithelial cells were isolated from each sample by scraping with a sterile lifter. Isolated cells were added to 100 μ L of cell lysis buffer consisting of 35mM Tris-HCl (pH 7.4), 0.2mM phenylmethyl-sulfonyl fluoride (PMSF), 10mM MgCl₂, 10mM 2-mercaptoethanol, 1 μ g/ml aprotinin and 5 μ g/ml Leupeptin. Cells were centrifuged at 10,000 $\times$ g for 30min at 4°C, and PKC activity was measured in both cytosolic and particulate cell fractions. The cell fractions were assayed as previously described (Wyatt et al., 2002) for changes in total PKC activity translocated from the cytosolic fraction to the particulate fraction. Kinase activity was expressed in relationship to total cellular protein assayed and calculated in pmol/minutes/mg. All samples were assayed in triplicate and no less than three separate experiments were performed per unique parameter.

Statistical Methods

Paired t-tests and post-hoc testing were used to analyze the differences in the tracheal CBF measurements before and after isoproterenol treatment. PKC data were analyzed using one-way ANOVA. Differences between the numbers of rats with positive cultures of their lungs were compared by Fischer's Exact test. Differences in the numbers of bacteria at the various anatomical sites were determined by the Mann-Whitney Rank Sum test for smoke vs. sham rats eating the same diet. Differences among the ethanol diet groups within each exposure group were examined by the Kruskal-Wallis ANOVA on ranks. Correlations between the number of organisms in the rats' lungs and % EtOH in their diets were determined by Spearman Rank Order Correlation. All statistical comparisons were made using either Graph Pad Prism (San Diego, CA) or SigmaStat (SPSS, Inc., Chicago, IL), and a *p* value of <0.05 was considered significant.

RESULTS

Blood Ethanol Levels

Blood alcohol levels at the end of weeks 1 and 3 of EtOH feeding are shown in Table 1. Smoke exposure did not significantly alter the blood alcohol levels, for rats eating any of the EtOH diets. Only those rats consuming diets containing the two highest EtOH concentrations had reliably measurable levels of EtOH in their blood (assay limit of detection is 10mg/dL).

Effect of Ethanol Ingestion and/or Smoke Exposure on Pneumococcal Movement into the Lower Respiratory Tract

Table 2 demonstrates that, regardless of smoke exposure, rats consuming higher concentrations of EtOH in their diets had a greater likelihood of pneumococci moving down their tracheas and into their lungs. The small number of culture-positive rats precluded statistical significance between diet groups by Fisher's Exact test. However, none of the smoke- or sham-exposed rats eating the 0% diet had organisms recovered from their lungs, whereas the number of rats with positive lung cultures increased with the amount of EtOH in their diets.

The number of organisms isolated from the rats' lungs tended to be higher in the smoke-exposed animals and to increase with the amount of EtOH in the diet (Figure 1). This increase in cfu's in the lungs correlated significantly with the percentage of EtOH in the diet for both the smoke-exposed ($p = .049$), and sham-exposed animals ($p = .018$).

Effect of Smoke Exposure and/or EtOH Ingestion on CBF

Within each of the diet groups, mean baseline CBF values were higher for smoke-exposed than sham-exposed animals, although none of the differences were statistically significant (Figure 2). Smoke exposure alone significantly diminished the ciliary response, and a negative change in CBF was seen in smoke-exposed animals consuming any of the EtOH-containing diets. It also was noted that the numbers of ciliated cells appeared somewhat diminished in the smoke-exposed animals, but quantitative measurements were not performed.

After stimulation with the β -agonist isoproterenol, the only rats that demonstrated a normal robust increase in CBF were the sham-exposed animals consuming the 0% EtOH diet. The isoproterenol response was diminished or even abolished in rats consuming any amount of EtOH in their diet, even the lowest EtOH concentration (16%) that did not result in elevated blood EtOH levels. CBF values in smoke-exposed and EtOH-fed rats were actually lower after stimulation than their baseline values, a phenomenon that was intensified as the amount of EtOH in the diet increased. Sham-exposed animals also exhibited an EtOH-induced blunting of the response to isoproterenol, although the stimulated value was only lower than the baseline for rats consuming the 36% EtOH diet.

Effect of Smoke Exposure and/or EtOH Ingestion on Protein Kinase Activity

While we have already demonstrated that chronic ethanol ingestion decreases PKA activity *in vivo* (Wyatt et al., 2004), no significant differences in PKA activity were shown between sham- and smoke-exposed rats within any of the EtOH feeding groups (data not shown). Likewise, smoke exposure alone failed to alter PKA activity in this experiment. By contrast, an equivalent amount of baseline PKC activity was detected in all the sham-exposed and smoke-exposed rats, regardless of the percentage of EtOH in their diet (Figure 3). Moreover, smoke-exposed rats in all EtOH feeding groups demonstrated a comparable and significant elevation in tracheal epithelial cell PKC activity in comparison to sham-exposed rats in the same feeding group (Figure 3). There were no significant changes in tracheal cell PKC activity in any of the groups of rats upon the addition of isoproterenol (data not shown).

DISCUSSION

There are many potential causes for increased susceptibility to pneumococcal pneumonia in people who abuse alcohol and/or smoke cigarettes. In the alcohol abuser it can be correlated with higher rates of aspiration of nasopharyngeal contents and a decreased cough response (Cooper and Maderazo, 1988; Preheim and Gentry, 1996). Smoking results in increased colonization and binding of *S. pneumoniae* to buccal epithelial cells, which increases the chance of exposure (). Both smoking and alcohol abuse also damage the function of the mucociliary clearance system, the major mechanical defense that prevents movement of organisms from the nasopharynx into the lower respiratory tract (Wolff, 1986).

We have utilized a rat model of concurrent smoke exposure and EtOH ingestion to quantify the separate and combined effects of these two insults on mucociliary clearance. This was accomplished by tracking the movement of *S. pneumoniae* from the nasopharynx into the lower respiratory tract over a four hour time course in EtOH-consuming and/or smoke-exposed rats. In addition, the CBF of tracheal epithelial cells from each rat was measured and the relationship between movement of bacteria and defects in mucociliary function was explored.

Ethanol ingestion alone resulted in increased movement of *S. pneumoniae* into the rats' tracheas and ultimately their lungs, as tracheal cultures were positive for all rats with positive lung cultures. This ethanol-induced increase in pneumococcal movement was evidenced in sham-exposed rats, but only in the two groups that had detectable blood alcohol levels. Smoke exposure alone did not increase movement of organisms into the lungs, but it did exacerbate the EtOH-induced increase in bacterial penetration, as well as extending it to rats in the 16% EtOH diet group that did not have reliably measurable blood alcohol levels. It is unclear from these studies whether a loss of cilia or ciliated cells from the airway epithelium contributed significantly to defective mucociliary clearance. Ciliary loss in response to cigarette smoke exposure has been documented both clinically () and in *in vitro* animal models (Sisson, et al., 1994). In the present study we observed a possible decrease in ciliated cells and beating cilia in tracheas from smoke-exposed animals. However, the small amount of available tracheal tissue precluded quantitative comparisons of ciliary dynein needed to precisely determine the amount of cilia lost in relationship to smoking-induced alterations in mucociliary function.

The ethanol- and smoke-induced defects in pneumococcal clearance also were associated with a decrease in the stimulatory response of the tracheal cilia. Ciliated cells isolated from the only groups of sham-exposed rats with positive lung cultures (those consuming the two highest concentrations of EtOH) exhibited a significant impairment in their ability to upregulate CBF after stimulation. These results corroborate earlier *in vitro* work showing that chronic EtOH exposure desensitizes tracheal epithelial cells to stimulation by isoproterenol (Wyatt and Sisson, 2001) and verify data from an earlier *in vivo* study with a smaller numbers of rats (Wyatt, et al., 2004). Smoke exposure in conjunction with consumption of any of the EtOH-containing diets dramatically decreased the ability of ciliated cells to respond to isoproterenol. It also resulted in a dose-dependent increase in movement of organisms into the lungs, both in terms of the number of rats with positive cultures and the mean numbers of organisms present in the lungs.

As shown earlier (Wyatt et al., 2004), the loss of stimulatory ability of ciliated cells from smoke-exposed and EtOH-fed animals was due to an EtOH-induced defect in the signal pathway between the stimulant and PKA activity in these cells. In the current study, baseline PKC levels also were increased in smoke-exposed whole animals, similar to what we have observed *in vitro* (Wyatt et al., 1999), but no activation of PKC was demonstrated in response to isoproterenol. Unlike the alpha-adrenergic stimulation of chloride transport via PKC (Liedtke, 1995), there is no clear evidence that beta agonists such as isoproterenol activate PKC in airway epithelial cells. While isoproterenol was shown to stimulate the elevation of cellular diacylglycerol in airway epithelium, no resultant activation of PKC was measured (Anderson and Welsh, 1990). Conversely, Penn, et al. reported that specific inhibitors of PKC failed to block the desensitization of the beta-agonist receptor (Penn, et al., 1998). Since activators of PKC have been shown to stimulate beta agonist receptor desensitization (Kelsen, et al., 1995), our observations suggest that whole animal smoke exposure may facilitate the down-regulation of isoproterenol responsiveness in the airways.

While the mechanism of cigarette smoke mediated decreases in CBF is not fully understood, we have shown that smoke stimulates the release of interleukin-8 in a PKC alpha-dependent manner (Kashyap et al., 2002), and have recently demonstrated that interleukin-8 is capable of autocrine inhibition of the β -agonist-stimulated CBF response (Allen-Gipson et al., 2004). Therefore, the additional desensitization of stimulated CBF observed with both smoke and ethanol may be mediated via the ability of smoke to stimulate cytokine release in a PKC-dependent manner in conjunction with the previously observed PKA desensitization by ethanol (Wyatt et al., 2004).

The present studies point to alterations of ciliary beat frequency and the mucociliary clearance mechanism as one potential cause of the increased risk of development of pneumococcal pneumonia in the smoking alcoholic population. Future studies using a longer time course are planned to further track the progression of the organisms into the lower respiratory tract and determine how alterations in bacterial movement relate to pneumonia development and severity.

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Figure 1.

Log of cfu/ml of *S. pneumoniae* isolated from lung tissue of individual smoke-exposed [A] and sham-exposed [B] rats (n = 8–9). Rats were sacrificed 4 h after intranasal infection with 1×10^8 cfu of type 3 *S. pneumoniae*. The lungs were removed and homogenized in 10 ml of PBS, and bacteria were enumerated by standard plate counts. Horizontal bars represent mean of the log values with samples having 0 cfu being counted as 0 for graphing and statistical purposes.

Figure 2.

Computerized spectrum analysis of CBF performed on tracheal ring explants ($n = 8-9$). Unstimulated (baseline) measurements were recorded immediately on a thermostatically controlled microscope. Samples then were treated with the β -agonist isoproterenol and stimulated CBF was determined. * Stimulated value for 0% diet group significantly greater than 26% and 36% diet groups within the same exposure group ($p < 0.001$ by ANOVA). † Sham stimulated value significantly greater than sham unstimulated value ($p < 0.001$ by t-test). ‡ Sham stimulated value greater than smoke stimulated value ($p = 0.007$ by t-test).

Figure 3.

Baseline PKC activity in cigarette smoke-exposed (black bars) or sham-exposed (white bars) rats consuming different concentrations of EtOH. Tracheal epithelial cells were extracted and assayed for PKC activity in the particulate fraction of the cells. Bars represent SEM of tracheal epithelial cell PKC activity from six different rats normalized against total cellular protein (* $p < 0.05$ for smoke-exposed vs. sham-exposed rats).

Table 1Blood Alcohol Levels in mg/dL $\pm$ SEM (n=8–9)

EtOH in Diet	Week 1		Week 3	
	Smoke-Exposed	Sham-Exposed	Smoke-Exposed	Sham-Exposed
36%	123.5 $\pm$ 2.4 *	109.6 $\pm$ 59.1 *	148 $\pm$ 66 *	165.2 $\pm$ 70.5 *
26%	31.8 $\pm$ 48.2	32.8 $\pm$ 41	34.4 $\pm$ 47.1	11 $\pm$ 25
16%	4.4 $\pm$ 8.1	3.3 $\pm$ 5.2	6.0 $\pm$ 8.8	3.2 $\pm$ 7.6
0%	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.7 $\pm$ 3.2

* 36% diet group significantly higher than all other diet groups within the same exposure group ($p < 0.001$).

Revised Table 2
EtOH Ingestion and Smoke Exposure Augment *S. pneumoniae* Movement

% EtOH in Diet	A. Respiratory Tract Colonization of Smoke-Exposed Rats					
	Distal Trachea		Proximal Trachea		Lungs	
	#Rats Colonized/ Total	Geometric Mean of cfu/ml*	# Rats Colonized/ Total	Geometric Mean of cfu/ml*	# Rats Colonized/ Total	Geometric Mean of cfu/ml*
36%	7/9	562.4	4/9	32.6	4/9	10.3
26%	5/9	61.8	4/9	19.3	3/9	4.8
16%	5/9	112.8	4/9	19.8	3/9	2.6
0%	3/9	14.9	2/9	3.8	0/9	0
% EtOH in Diet	B. Respiratory Tract Colonization of Sham-Exposed Rats					
	Distal Trachea		Proximal Trachea		Lungs	
	#Rats Colonized/ Total	Geometric Mean of cfu/ ml*	# Rats Colonized/ Total	Geometric Mean of cfu/ ml*	# Rats Colonized/ Total	Geometric Mean of cfu/ ml*
36%	4/9	69.4	4/9	24.9	3/9	4.6
26%	5/9	207.5	5/9	17.4	1/9	1.1
16%	4/8	70.8	2/8	2.4	0/8	0
0%	3/8	16.4	2/8	5.3	0/8	0

* Antilog of mean log cfu/ml in fluid or homogenate