Ethanol Ingestion Reduces Antipneumococcal Activity of Rat Pulmonary Surfactant

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Because chronic ethanol ingestion decreases pulmonary clearance of *Streptococcus pneumoniae* in rats, and extracellular antipneumococcal factors in rat surfactant are important in the early clearance of pneumococci from the rat alveolus, the effects of ethanol ingestion on surfactant bactericidal activity were investigated. Normal surfactant from chow-fed rats showed potent antipneumococcal activity, even against bacteria growing in nutrient-rich media under favorable conditions. In contrast, surfactant from ethanol-fed rats and from calorie-restricted control-fed rats had significantly reduced antipneumococcal activity compared with surfactant from chow-fed rats. The reductions in surfactant bactericidal activity produced by ethanol ingestion or caloric restriction did not appear to be mediated through changes in either the total amount or the distribution of fatty acids, the antipneumococcal factors in normal surfactant. Rather, ethanol ingestion, and to a lesser extent caloric restriction, produced a surfactant inhibitor of free fatty acids that was partially characterized as a hydrophobic protein.

Ethanol abuse is a major risk factor for severe and invasive *Streptococcus pneumoniae* pulmonary infection, particularly among young adults [1]. Alcoholics have a higher incidence of complicated pneumococcal pneumonia, with pulmonary necrosis, cavitation, and empyema [2]. Furthermore, bacteremic pneumonia has a case-fatality rate of up to 70% in alcoholics [3, 4].

Although ethanol ingestion profoundly impairs many aspects of systemic host defense against bacterial infection, the relationship of these effects to the pathogenesis of pneumococcal pneumonia is poorly defined. In a rat model of pneumococcal pneumonia, chronic ethanol ingestion significantly increases the severity of pulmonary infection, bacteremia, and mortality [5]. Although the net clearance of pneumococci from the lung is decreased in ethanol-treated rats compared with control rats, ethanol ingestion does not significantly impair the cellular pulmonary defense mechanisms in treated rats. Specifically, chronic ethanol ingestion does not significantly reduce activation of the complement system [5], the recruitment of neutrophils to lungs during pneumonia [6], or opsonophagocytosis and killing of pneumococci [7]. Taken together, these studies suggest that ethanol ingestion might reduce clearance of pneumococci from murine lungs by impairing the bactericidal activity of extracellular pulmonary factors.

Cell-free rat pulmonary surfactant has potent bactericidal activity against *S. pneumoniae*, primarily through a detergent-like action of surfactant long-chain free fatty acids (FFAs) [8]. Rat surfactant FFAs are produced in the lung through the hydrolysis of triglycerides by macrophage-derived lipoprotein lipase [9]. Because rat cardiac lipoprotein lipase activity is decreased by ethanol administration [10], we speculated that ethanol might decrease bactericidal surfactant FFAs through inhibition of lung lipoprotein lipase. Therefore, we investigated the effects of ethanol ingestion on the antipneumococcal activity of rat pulmonary surfactant.

**Materials and Methods**

*Animals and feeding protocol.* Pairs of rats were administered ethanol or control diets as described [5]. Briefly, male rats (Sasco, Omaha) were housed in group cages and fed standard rat chow and water until they reached an average weight of 150 g. Rats were paired by weight, placed in individual cages, and acclimated to the liquid Lieber-DeCarli control diet (Dyets, Bethlehem, PA) for 3 days. One rat of each pair received Lieber-DeCarli diet providing 36% of calories in the form of ethanol (ethanol-fed), while the second rat received the amount of isocaloric control diet equivalent to that consumed by its ethanol-fed mate on the previous day (control-fed). A third group of rats (chow-fed) received standard rat chow and water ad libitum. Rats were maintained on their diets for 7–10 days before being sacrificed for isolation of pulmonary surfactant.

*Bronchoalveolar lavage and isolation of pulmonary surfactant.* Bronchoalveolar lavage was done on day 7–10 as described [8]. Briefly, rats were killed by lethal injection of sodium pentobarbital intraperitoneally, and the lungs were lavaged in situ through a plastic catheter secured in the trachea. After lavage with 5 mL of Dulbecco’s PBS for two or three cycles, the lavage fluid was centrifuged at 600 g for 10 min at 4°C to remove cells. Cell-free supernatants from samples from 6 rats were pooled, and pulmonary...
surfactant was pelleted by recentrifuging the pooled supernatants at 55,000 g for 30 min. Pelleted surfactant was concentrated by resuspension in PBS at 1/20 of the original volume and was frozen in aliquots at -70°C until assayed.

**Bacterial growth curves.** Type 2, 3, and 14 S. pneumoniae (strains 6302, 6303, and 6314; American Type Culture Collection, Rockville, MD) were grown to log phase in brain-heart infusion (BHI) broth. After being washed in PBS, bacteria (~10^7 cfu) were then inoculated into 1 mL of fresh BHI broth containing varying concentrations of PBS or surfactant samples and incubated at 37°C in 5% CO_2_. At selected times, aliquots (100 μL) of broth were serially diluted in PBS and quantitatively cultured on 5% sheep blood agar plates.

**Lipid extraction and thin-layer chromatography (TLC).** Lipids were extracted from pooled pulmonary surfactant with chloroform-methanol using a modified Bligh-Dyer procedure [11]. FFAs were separated from neutral lipids by one-dimensional preparative TLC on silica G 60 plates (Analtech, Newark, NJ) with a solvent system consisting of ether–hexane–glacial acetic acid (50:50:1). Chromatograms were developed with authentic standards (Nu-Check Prep, Elysian, MN), and spots were identified by iodine vapor. A rectangle, appropriate to the size of each visualized standard, was scraped into glass tubes to be reextracted with chloroform-methanol (2:1) and dried under nitrogen. Extracted lipids and FFAs were resuspended in BHI broth by sonication for assay of bactericidal activity.

**Gas-liquid chromatography.** Pulmonary surfactant was trans-esterified [12] and the fatty acid methyl esters separated and quantified by gas-liquid chromatography using a model 5890 series II gas chromatograph fitted with a 30-m × 0.25-mm HP-INNOWAX (cross-linked polyethylene glycol) 0.25-μm film thickness capillary column (Hewlett-Packard, Palo Alto, CA). Pentadecanoic acid (C15:0) was added and used as an internal standard.

**FFA inhibitor assay.** Palmitoleic acid (Sigma, St. Louis) was dried under nitrogen and resuspended by sonication at 50 μg/mL in BHI broth. After surfactant samples or PBS controls were mixed with palmitoleic acid suspensions, a quantity of BHI broth sufficient to bring the volume to 1 mL was added, and the mixture was inoculated with type 3 S. pneumoniae (3 × 10^6 cfu/mL). After incubation for 5 h at 37°C under 5% CO_2, bacterial colony-forming units per milliliter of mixture was determined by quantitative culture on 5% sheep blood agar plates. Percentage of bactericidal activity was calculated as the percentage reduction in bacterial growth after incubation with the sample, compared with the reduction in bacterial growth produced by palmitoleic acid.

**Protease digestion.** Samples of ethanol-fed rat surfactant or PBS were incubated with 100 U of insoluble Staphylococcus aureus V8 protease (Sigma) at 37°C for 60 min, then were separated from protease by centrifugation at 2000 g for 10 min before use in experiments.

**Albumin depletion and blocking.** To deplete albumin in surfactant samples, ethanol-fed rat surfactant or PBS was incubated for 60 min at 37°C with goat IgG to rat albumin (Organon Teknika/Cappel, West Chester, PA) immobilized on agarose beads. Albumin-depleted surfactant was separated from immobilized antibodies by centrifugation at 2000 g for 10 min before use in experiments. Albumin depletion was confirmed by analysis of surfactant proteins by SDS-PAGE and Coomassie staining. In blocking experiments, ethanol-fed rat surfactant was preincubated with goat IgG to rat albumin (1:100) for 30 min at 37°C before assay of FFA inhibitor activity.

**Statistics.** Geometric means of bacterial colony-forming units were compared by one-way analysis of variance and, when significantly different (P < .05), by post hoc Bonferroni t test, using SPSS for Windows 6.1 (SPSS, Chicago). Data points in figures represent the geometric mean and SD of 3–10 repeat experiments.

**Results**

**Effect of pulmonary surfactant on pneumococcal growth.** Pulmonary surfactant from normal rats accelerates the death of large inocula of S. pneumoniae over several hours in nutrient-poor buffers such as PBS [8]. However, pneumococci auto- lyze substantially in nutrient-poor buffers over several hours and thus may be more susceptible to surfactant bactericidal factors under these adverse conditions. Therefore, to confirm that normal rat pulmonary surfactant was also bactericidal to pneumococci actively growing in a nutrient-rich medium, type 3 S. pneumoniae in log-phase growth were inoculated at 2 × 10^7 cfu/mL into BHI broth containing either 20% (vol/vol) surfactant from chow-fed rats or 20% (vol/vol) PBS, and aliquots were removed at 2-h intervals for quantitative culture.

Consistent with previous studies [8], rat pulmonary surfactant at these concentrations markedly inhibited net pneumococcal growth for 4 h and produced a rapid decline in bacterial numbers 4–8 h after inoculation (figure 1A). Surfactant bactericidal activity was concentration-dependent, with detectable effects at 2.5% (vol/vol) surfactant in BHI broth and maximal effect at 50% surfactant in BHI broth (the highest concentration tested) (figure 1B). At maximal concentrations, rat surfactant rapidly killed pneumococci, with bacterial numbers reduced by 1 log at 45 min and by 4 logs at 2 h (data not shown). In addition, rat surfactant had comparable bactericidal effects on serotypes 2 and 14 S. pneumoniae (data not shown).

Surfactant FFAs are bactericidal to pneumococci inoculated into nutrient-poor buffers [8]. To verify that the rat surfactant FFAs were responsible for the antipneumococcal activity observed in our assay, we extracted surfactant lipids and separated FFAs by preparative TLC. The bactericidal activity was quantitatively recovered in the organic phase after lipid extraction and purified with the FFA fraction after TLC (data not shown). Thus, FFAs in normal rat surfactant were bactericidal to S. pneumoniae even under nutrient-rich conditions, such as may occur during natural lung infection.

**Effect of ethanol and control liquid diets on surfactant bactericidal activity.** Because ethanol-fed rats have decreased net pulmonary clearance of pneumococci during experimental pneumonia, we investigated whether the surfactant antipneumococcal activity might be reduced in these animals. Samples of ethanol- and chow-fed rat surfactant had comparable protein (220 vs. 235 μg/mL) and total phosphorus (3.4 vs. 3.8 μg/mL) concentrations. However, in marked contrast to surfactant from chow-fed rats, 25% (vol/vol) pulmonary surfactant from etha-
of nutrition for the 10-day experimental period. Ethanol-fed rats limited their consumption of the ethanol diet to ~35 mL/day (35.4 ± 3.4 mL), which provided ~35 Kcal/day (Lieber-DeCarli diet = 1 Kcal/mL). Chow-fed rats had unrestricted access to their pelleted diet and consumed 3.5 times more calories each day (122.2 ± 3.6 Kcal/day) than ethanol-fed rats. To control for the caloric restriction of the ethanol diet, control-fed rats received the same number of calories in an equivalent volume of isocaloric liquid control diet as the ethanol-fed rats consumed each day.

Using this feeding paradigm, ethanol- and control-fed rats grew more slowly than chow-fed rats. Both ethanol-fed and control-fed rats maintained their weights at 163 ± 2 g for 10 days, whereas chow-fed rats increased their weight to 263 g during this period. Similar to surfactant from ethanol-fed rats, 25% (vol/vol) pulmonary surfactant from control-fed rats had reduced bactericidal activity compared with surfactant from chow-fed rats (figure 2). Pneumococci incubated in control-fed rat surfactant tended toward less robust growth than bacteria in surfactant from ethanol-fed rats, although these differences approached but did not meet statistical significance (P = .1). Therefore, ingestion of ethanol or caloric restriction decreased the bactericidal activity of pulmonary surfactant, suggesting that the effects of ethanol are at least partially attributable to concomitant caloric restriction.

Effect of diet on pulmonary surfactant FFAs. Pulmonary surfactant long-chain FFAs, particularly the unsaturated FFAs arachidonic, palmitoleic, linoleic, and oleic acids, are the bactericidal factors in normal rat surfactant [8]. To determine

![Figure 1](image1.png)

**Figure 1.** Bactericidal activity against type 3 *Streptococcus pneumoniae* of surfactant from chow-fed rats: time course (A) and concentration dependence (B). Data points = geometric means + SD of 3 experiments. * P < .05 vs. PBS.

tanol-fed rats fully supported growth of type 3 *S. pneumoniae* in BHI broth (figure 2). Compared with surfactant from chow-fed rats, bacterial colony counts of pneumococci growing in surfactant from ethanol-fed rats were ~3 logs higher at 3 h and >4 logs higher at 6 h after inoculation. Thus, ethanol ingestion for 7–10 days appeared to significantly reduce the bactericidal activity of rat pulmonary surfactant.

Because of the natural aversion of rats to ethanol, ethanol is administered to rats as a liquid diet that is the sole source

![Figure 2](image2.png)

**Figure 2.** Effect of diet on bactericidal activity of rat surfactant from rats fed ethanol (EtOH), chow, or a calorie-restricted control diet. Data points = geometric means + SD of 6 experiments. * P = .02 vs. PBS.
whether the reductions in bactericidal activity correlated with changes in surfactant FFAs from ethanol- and control-fed rats, pooled samples of surfactant from each group of rats were analyzed by gas chromatography for fatty acid composition. Surfactant from ethanol-fed rats contained significantly more fatty acids than did that from chow- or control-fed rats (530.4 ± 43.9, 282.7 ± 61.6, and 362.3 ± 32.0 μg/animal, respectively; *P < .05). Although there were no significant differences in the proportions of fatty acids in surfactant from the 3 groups, the proportion of unsaturated fatty acids was actually slightly higher in surfactant from ethanol-fed rats than in that from chow-fed rats (table 1). Thus, the reduced antipneumococcal activity of surfactant from ethanol- and control-fed rats did not correlate with decreased amounts of bactericidal fatty acids in these surfactants.

To verify that the differences in the antipneumococcal activities of the surfactants was not explained by their respective fatty acid compositions, we assayed the bactericidal activity of mixtures of purified FFA standards reconstituted in the same concentrations found in surfactant from chow-, ethanol-, and control-fed rats. In contrast to the marked differences in antipneumococcal activities of the surfactants from the 3 groups, these reconstituted FFA mixtures all were potently bactericidal at concentrations equivalent to 5%-50% (vol/vol) surfactant in BHI broth (data not shown). Taken together, these results suggested that the reduced bactericidal activity of ethanol- and control-fed rat surfactants was not explained by differences in their total amounts of FFA or in their FFA compositions.

Identification of surfactant inhibitor of FFA antipneumococcal activity. Because the decreased antipneumococcal activity of ethanol-fed rat surfactant was not explained by reduced amounts of bactericidal FFAs, we investigated whether ethanol-fed rat surfactant might contain an inhibitor of this activity. Initial mixing studies demonstrated that combinations of chow- and ethanol-fed rat surfactant lacked antipneumococcal activity (data not shown), suggesting that a component of ethanol-fed rat surfactant could inhibit the bactericidal FFAs present in chow-fed rat surfactant. To further characterize this inhibitor,

Table 1. Fatty acid composition of rat pulmonary surfactant.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Chow-fed</th>
<th>Ethanol-fed</th>
<th>Control-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic (14:0)</td>
<td>2.4 ± 1.2</td>
<td>1.7 ± 0.5</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>52.9 ± 6.5</td>
<td>51.1 ± 6.3</td>
<td>60.2 ± 1.4</td>
</tr>
<tr>
<td>Palmitoleic (16:1)</td>
<td>9.4 ± 4.3</td>
<td>15.2 ± 3.2</td>
<td>9.9 ± 0.2</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>6.2 ± 3.7</td>
<td>3.9 ± 0.5</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>8.6 ± 4.1</td>
<td>12.4 ± 3.5</td>
<td>8.7 ± 0.4</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>3.4 ± 1.8</td>
<td>4.8 ± 1.9</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>Arachidonic (20:4)</td>
<td>1.1 ± 1.2</td>
<td>2.2 ± 1.2</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Other*</td>
<td>0</td>
<td>0.9 ± 1.5</td>
<td>2.4 ± 0.6</td>
</tr>
</tbody>
</table>

NOTE. Data are % of total (mean ± SD).

* α-linolenic and decosahexaenoic acids.

samples of chow- and ethanol-fed rat surfactant were assayed for inhibition of the antipneumococcal activity of purified palmitoleic acid. Incubation of type 3 S. pneumoniae with 5 μg/mL palmitoleic acid in BHI broth reduced bacterial numbers by 6 logs at 5 h. Addition of 10% ethanol-fed rat surfactant almost completely inhibited this bactericidal activity of palmitoleic acid, whereas 10% chow-fed rat surfactant produced minimal inhibition of FFA activity and 10% control-fed surfactant had intermediate effects (table 2). The FFA inhibitor in ethanol-fed rat surfactant was highly sensitive to treatment with insoluble staphylococcal V8 protease, was largely resistant to boiling for 5 min, was not affected by blocking or depleting albumin, and partitioned with the organic phase after chloroform-methanol extraction (table 2). Titration of inhibitor activity against increasing concentrations of FFAs confirmed that ethanol-fed rat surfactant had the highest amounts of inhibitor, whereas control-fed rat surfactant had intermediate amounts and chow-fed rat surfactant had the lowest amounts (figure 3).

Discussion

We have demonstrated that chronic ethanol ingestion significantly reduces the antipneumococcal activity of rat surfactant. Similar to its effects in humans, ethanol intoxication for 7–10 days in this murine model significantly increases the severity of pneumococcal pneumonia and the incidence of inva-
Figure 3. Titration of inhibitor activity against free fatty acid (FFA) in surfactant from chow-fed, control-fed, or ethanol (EtOH)-fed rats. Data points = geometric means + SD of 3 experiments. * P < .05 vs. chow.

Ethanol ingestion and caloric restriction appeared to reduce surfactant bactericidal activity by producing an inhibitor of surfactant FFAs, rather than by affecting the total amounts or distribution of bactericidal FFAs. No significant differences in total lipids or FFAs were detected in surfactants from ethanol- or control-fed rats compared with chow-fed rats. Comparison of the antipneumococcal activity of mixtures of purified FFAs reconstituted in the same proportions found in ethanol-, control-, and chow-fed rat surfactants revealed no differences in the antipneumococcal activities of these three FFA mixtures. Therefore, we concluded that the reduced bactericidal activities of ethanol- and control-fed rat surfactant could not be explained primarily by changes in surfactant FFAs.

Through mixing studies and assay of inhibition of the antipneumococcal activity of purified FFAs, we demonstrated increased amounts of an FFA inhibitor in ethanol- and control-fed rat surfactant compared with chow-fed rat surfactant. Tilters of the FFA inhibitor in surfactant from the 3 groups correlated with the reductions in antipneumococcal activity of the respective surfactants. Therefore, ethanol and control diets appeared to reduce surfactant antipneumococcal activity by increasing levels or enhancing binding of a surfactant inhibitor of bactericidal FFAs.

Further characterization revealed that the FFA inhibitor in surfactant was heat-stable, protease-sensitive, and hydrophobic. Surfactant contains albumin and IgG in low amounts and four surfactant-specific proteins (SP), the hydrophilic proteins SP-A and SP-D and the hydrophobic proteins SP-B and SP-C [16]. Although albumin is a major FFA-binding protein in serum, the physical properties of the surfactant inhibitor as well as our inability to neutralize the inhibitor with anti-rat albumin antibodies indicated that this FFA inhibitor was distinct from albumin. Furthermore, this FFA inhibitor appeared distinct from an aqueous, low-molecular-weight FFA inhibitor described in neonatal rat surfactant, which also has reduced antipneumococcal activity compared with adult rat surfactant [17]. Although their ability to bind FFAs has not been reported, SP-B and SP-C are the hydrophobic surfactant proteins that, along with SP-A and SP-D, combine with phospholipids to markedly reduce the surface tension of lung surfactant. We speculate that surfactant proteins can similarly bind FFAs and that ethanol ingestion and caloric restriction either increase the total amount of hydrophobic surfactant proteins or the FFA-binding capacity of SP-B or SP-C.

In summary, ethanol ingestion, and to a lesser degree caloric restriction, reduces the antipneumococcal activity of rat lung surfactant primarily by increasing amounts of a hydrophobic surfactant protein inhibitor of FFAs. Along with other patho-

sive pneumococcal infection, apparently by decreasing bacterial clearance from the lung [5]. Extracellular bactericidal factors appear to be particularly important in the early clearance of pneumococci from the rat alveoli, killing up to 90% of inhaled pneumococci within 60 min after deposition in the lung [13]. Although factors including lysozyme and transferrin contribute to this extracellular bactericidal activity [13, 14], long-chain unsaturated FFAs in rat surfactant are the major source of antipneumococcal activity in alveolar lavage fluid [8]. Previous studies have shown that surfactant FFAs are bactericidal to pneumococci growing in nutrient-poor buffers [8]. Our studies corroborate that surfactant FFAs from rats on standard lab chow diets are bactericidal to pneumococci, even when bacteria are grown under favorable conditions in nutrient-rich media, similar to those in alveoli flooded with serum during natural infection.

In contrast, ethanol ingestion for 7–10 days totally abolished the surfactant bactericidal activity, thus eliminating a major early lung clearance mechanism and promoting intrapulmonary growth and dissemination of pneumococci during pneumonia. Many of the pathogenic effects attributed to ethanol ingestion in humans may be related to the associated reduced caloric intake and nutrition [15]. Surfactant from control-fed rats with caloric intake restricted to that observed in pair-matched ethanol-fed rats also showed significantly reduced antipneumococcal activity compared with surfactant from chow-fed rats. Compared with ethanol-fed rat surfactant, control-fed rat surfactant appeared to have some residual antipneumococcal activity, although the differences between bacterial growth in ethanol- and control-fed rat surfactant did not meet statistical significance. Thus, much of the effect of ethanol ingestion on surfactant in this model appeared secondary to reduced caloric intake, but the effects from ethanol itself might be additive.
genic effects of ethanol ingestion, this reduction in the bactericidal activity of rat surfactant may decrease the extracellular alveolar clearance of bacteria during the early stages of pneumococcal pneumonia and thereby increase the severity of pneumonia and the incidence of invasive pneumococcal infection.

Acknowledgments

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References