Perfluorooctyl Bromide (Perflubron) Stimulates Mucin Secretion in the Ferret Trachea*

Chikako Kishioka, MD, PhD; Matthew P. Dorighi, MD; and Bruce K. Rubin, MD, FCCP

**Objectives:** Partial liquid ventilation with perfluorooctyl bromide (perflubron) has been shown to be safe and effective in animal models with respiratory failure. However, airway mucus accumulation has been reported to be a problem in human trials. We hypothesized that this might be because perflubron directly affects mucociliary clearance or stimulates mucus secretion.

**Methods and results:** We first measured the mucociliary transportability of secretions on the mucus-depleted frog palate exposed to perflubron and demonstrated that the ciliated epithelium remained intact with preservation of mucociliary transport. We then measured mucin and lysozyme secretion from isolated ferret tracheal segments to evaluate the secretagogue potential of perflubron. There was an 86% increase in mucin secretion with perflubron incubation at 40 min (n = 19; p < 0.01) and a 52% increase after 4 h of exposure followed by evaporation of perflubron (n = 19; p < 0.01). There was no significant difference in lysozyme secretion at any time between perflubron-exposed or buffer-exposed tissue (n = 4). The secretagogue effect was completely blocked by nordihydroguaiaretic acid, an inhibitor of arachidonic acid (AA) metabolism.

**Conclusion:** These data suggest that although perflubron does not seem to be harmful to the airway, it induces mucus secretion in a noninflamed airway, and that this can be modulated by inhibitors of AA metabolism.

**Key words:** partial liquid ventilation; mucociliary clearance; secretagogue; arachidonic acid; mucin; lysozyme

**Abbreviations:**
- AA = arachidonic acid
- DBA = Dolichos biflorus agglutinin
- ELLA = enzyme-linked lectin assay
- KHS = Krebs-Henseleit solution
- MARS = modified amphibian Ringer's solution
- MCTR = mucociliary transportability
- NDGA = nordihydroguaiaretic acid
- PBS = phosphate-buffered saline
- PIC = protease inhibitor cocktail
- PLV = partial liquid ventilation
- SI = secretory index

Perfluorooctyl bromide (perflubron) was first developed in the early 1970s as a contrast material for bronchography. Perflubron has a high oxygen-carrying capacity, positive spreading coefficient, and low surface tension. Partial liquid ventilation (PLV) with perflubron has been shown to be safe and effective for the therapy of respiratory failure in animals.

However, in clinical trials, some patients treated with PLV developed airway obstruction due to mucus plugging. Increased mucus accumulation has been reported especially at the induction and at the end of PLV. These secretions can usually be removed by frequent suctioning.

There are several possible mechanisms to explain mucus accumulation in patients treated with PLV. Perflubron might increase the clearance of preformed secretions because of its high density and low surface tension. Excessive secretions during and immediately after PLV might also be due, in part, to perflubron inducing mucus secretion directly or through the generation of mediators that induce secretion.

In this study, we wished to evaluate whether the ciliated epithelium remains intact with physiological preservation of mucociliary transport after per-
flubron exposure and to assess whether or not perflubron induces mucus secretion.

Materials and Methods

Study Design

Mucociliary Transportability of Secretions: To determine whether perflubron affects the mucociliary interaction, we first evaluated the mucociliary transportability of secretions (MCTR) on the mucus-depleted frog palate. Sixteen mature northern leopard frogs were randomly separated into two groups of eight frogs. The control group palpates were incubated with modified amphibian Ringer’s solution (MARS), and the perflubron group was exposed to perflubron as detailed later. We measured MCTR before exposure to perflubron or MARS after immersion times of 5 min and 18 h.

Evaluation of Secretagogue Potential: To evaluate whether perflubron induced mucus secretion, secretion from mucous cells and serous cells in the ferret trachea was evaluated by determining mucin and lysozyme secretion into the bathing solution. After sacrifice, tracheal removal, and a standard 2-h recovery period, ferret tracheal segments were incubated in Krebs-Henseleit solution (KHS) as a negative control, or perflubron for 40 min, or perflubron for 4 h, followed by drainage of the perflubron and 30 min of exposure to heated humidified room air to complete perflubron evaporation in the segment, followed by segment re-immersion in warmed KHS to collect all additional secretions. Evaluation of Secretory Pathways: To evaluate the possible pathways of mucin secretion, the tracheal segments were pretreated with specific inhibitors of stimulated secretion. After a recovery period, the segments were incubated for 40 min with each inhibitor in KHS, or with only KHS, then incubated for 40 min or for 4 h with perflubron or with KHS.

Methods

MCTR: The frog was pithed by bending the head forward and inserting an 18 gage needle into the brain and the spinal cord. The jaw was disarticulated and the palate removed. The excised palate was placed on a piece of gauze saturated with MARS and prepared by mixing 2 parts of non-lactated Ringer’s injection solution with 1 part sterile water. The palate was then placed to rest at 8°C for 12 to 18 h to deplete mucus. The palate was then placed in a chamber in which the relative humidity was maintained at 95 to 100% and the temperature was maintained at 22°C to 24°C. The palate was focused under a microscope so that a 12.7-mm micrometer scale ran between the optic buds to the opening of the esophagus. Measurement of MCTR was made using endogenous frog mucus. The movement of the frog mucus was timed as the trailing edge moved across a 7.62-mm segment. At least three measurements of mucus transport rate were taken to minimize variability, and the average transport rate was calculated.5 Evaluation of Secretagogue Potential: Adult ferrets (weight, 1.7 to 2.4 kg; random sex) obtained from Marshall Farms (North Rose, NY) were killed by an intraperitoneal injection of pentobarbital sodium, and the trachea from larynx to carina was immediately removed. Each trachea was divided into four roughly equal segments from the upper trachea to the carina. The segments were weighed and then immersed in 10 mL of KHS and agitated gently in a shaking water bath at 38°C. Oxygen was provided at 0.5 L/min. After a 2-h recovery time, the segments were incubated for 40 min in KHS (period 1). Tracheal segments were then incubated for 40 min or for 4 h with perflubron or with KHS (period 2). After the experiment, the KHS was analyzed for mucin and lysozyme content as described below. The perflubron-incubated segments were re-immersed in warmed KHS for 2 min to collect secretions. Ferret tracheal segments were also incubated in perflubron for 4 h, followed by drainage of the perflubron and 30 min of exposure to heated, humidified room air to complete perflubron evaporation. The segment was then re-immersed in warmed KHS for 2 min to collect secretions (period 2).

The relative contribution of mucous or serous cells to the secretions was evaluated by measuring the amount of mucin, which is a marker for mucous cell secretion, and lysozyme, which is of serous cell origin.10 A secretory index expressing the relative increase in secretion under experimental conditions was calculated for each tracheal segment as mucin or lysozyme secretion after 40 min of exposure to perflubron (period 2) divided by the amount secreted after 40 min of exposure to KHS alone (period 1).

We randomized the administration of perflubron and KHS to different segments in each experiment. A sufficient number of tracheas were studied to eliminate anatomic variation in secretion as a potential confounding variable when analyzing results. Evaluation of Secretory Pathways: The above protocol was also used to evaluate the physiological mechanism of mucin secretion with perflubron exposure, but tracheas were pretreated with specific inhibitors of stimulated secretion. After a 2-h recovery period, ferret tracheal segments were incubated for 40 min with each inhibitor in KHS or with KHS alone (period 1), then incubated for 40 min or for 4 h with perflubron or with KHS (period 2). For each potential inhibitor, five experiments were performed for each time period (40 min or 4 h).

Agents Used for Secretion Inhibition:

[1] Atropine (10⁻⁴ M) as a cholinergic antagonist⁷–⁹
[2] Phentolamine (10⁻⁴ M) as an α-adrenergic receptor antagonist⁷–¹⁰
[3] Propranolol (10⁻⁴ M) as a β-adrenergic receptor antagonist⁷–¹⁰
[4] Methylprednisolone (10⁻⁴ M and 10⁻⁵ M) as a phospholipase A₂ inhibitor¹¹–¹⁴ and nordihydroguaiaretic acid (NDGA) (10⁻⁴ M and 10⁻⁵ M), which is a combined cyclooxygenase and lipoxygenase pathway inhibitor.¹⁵–¹⁷
[5] A protease inhibitor cocktail (PIC) of phenylmethylsulfonyl fluoride as an inhibitor of serine protease, leupeptin as a nonspecific protease inhibitor, and pepstatin A as an acid protease inhibitor. We used this cocktail to block all proteinase activity.

Mucin Analysis by Sandwich Enzyme-Linked Lectin Assay: Ferret tracheal mucins have high titers for blood group A, reflecting an abundance of galactose-N-acetylated-α-1–3 (Fucose-α-1–2) galactose-R, and this antigen has been shown to be an excellent marker for ferret mucin secretion.¹⁸ A sandwich enzyme-linked lectin assay was used to quantify secretion using this antigen as previously described.⁹ The amount of mucin was calculated by comparison with standard mucin (asialo ovine submaxillary mucin). The change in mucin secretion after exposure to the test agent was expressed as percent increase above control in mucin weight per trachea tissue weight (ng/g). Each sample was measured four times and the results were averaged. Lysozyme Analysis by Spectrophotometry: Lysozyme is a bacterialidic enzyme found in airway fluid, and the only airway source of this enzyme is the serous cells of the submucous glands.¹⁹ Lysozyme activity was measured spectrophotometrically by measuring the rate of lysis of a 1.38 mg/mL Micrococcus lysodeikticus suspension. An 0.1 mL volume of sample buffer was added to 1.4 mL of substrate in a phosphate-buffered saline buffer at pH 6.0. The change in turbidity was measured at 540 nm. One unit of enzyme activity was defined as the change in absorbance per minute equivalent to that produced by 1.0 mg of
egg white lysozyme under identical assay conditions. The enzyme activity of the sample was calculated by comparison with standard egg white lysozyme. The change in lysozyme release after test-agent exposure was expressed as the percent increase above excipient control in units/tissue weight (g).

Data Analysis

Statistical analysis of data was performed using the StatView 4.5 statistics package (Abacus Concepts Inc.; Berkeley, CA). The data were analyzed using paired comparisons for normally distributed data where appropriate, and between-group analysis of variance for multiple comparisons. Conventionally, p values of ≤ 0.05 were considered statistically significant. All data are presented as the mean ± standard error of the mean.

Results

Mucociliary Transportability and the Effect of Perflubron on the Frog Palate Ciliated Epithelium

There was no significant change in MCTR between MARS-treated and perflubron-treated frog palates at any immersion time (Fig 1). Immersion in MARS and perflubron increased MCTR after 5 min, but this increase was not statistically significant. There was also no significant change in MCTR after 18 h of immersion when compared with time 0. There were no gross histological changes in the epithelium seen microscopically at the time that MCTR was measured, demonstrating that even after an 18-h exposure to perflubron, the ciliated epithelium remains intact with physiological preservation of mucociliary transport.

Evaluation of Secretagogue Potential

The measured values of mucin secretion fell within the range detected by the standard curve. There was an 86.4% increase in mucin secretion over control from ferret trachea incubated for 40 min in perflubron (p = 0.006), a 17.6% increase after 4 h of exposure to perflubron (p = 0.02), and a 52.4% increase after 4 h of exposure to perflubron followed by evaporation (p = 0.004) (Fig 2). Each of these experiments had n = 19 ferrets with 20 segments for KHS and 25 for perflubron. There was no significant difference in lysozyme secretion (n = 4) between perflubron- and KHS-exposed segments in any of the three experiments (40 min, 4 h, and 4 h + evaporation).

Because most (but not all) secretagogues induce glandular secretion from both mucous and serous cells equally, we questioned whether the perflubron was interfering with the enzymatically determined lysozyme assay. We therefore evaluated the activity of standard egg white lysozyme in different concentrations after incubation with perflubron and found no reduction in activity.

Evaluation of Potential Secretory Pathways Using Selective Inhibitors of Secretion

There was a 30% increase over control in mucin secretion after 40 min of incubation with perflubron (n = 10; p = 0.055) in this set of experiments. NDGA completely inhibited the secretory effect at 40 min (n = 5, p = not significant) compared with the sample incubated with perflubron for 40 min. None of the other inhibitors suppressed this effect (Fig 3).

After 4 h of exposure to perflubron followed by
evaporation, there was a 30% increase in mucin secretion \((n = 10, p = 0.031)\). Atropine (43%, \(n = 5\)) and methylprednisolone (45%, \(n = 5\)) partially inhibited the increase of mucin secretion, and NDGA (\(n = 5\)) and PIC (\(n = 5\)) completely inhibited the increase of mucin secretion, although the results were not statistically significant. Phentolamine and propranolol had no effect on the stimulated mucin secretion (Fig 4).

After 4 h of incubation and before evaporation of perflubron, there was a 20% increase in mucin secretion \((n = 10, p = \text{NS})\). NDGA and PIC completely inhibited this effect (100%, \(n = 5, p = \text{NS}\)).

**Figure 3.** With perflubron incubation after 40 min, there was a 30% increase in mucin secretion. NDGA completely suppressed the increased mucin secretion.

**Figure 4.** After 4 h of incubation followed by perflubron evaporation, there was a 30% increase in mucin secretion. Atropine and methylprednisolone partially (43% and 45%, respectively), and NDGA and PIC completely suppressed the stimulation of mucin secretion.

**Discussion**

We have previously demonstrated that surface tension-lowering agents (surfactants) can markedly increase mucociliary transport.\(^{20,21}\) Because perflubron has a lower surface tension than secretions, we hypothesized that there may be an increase in MCTR with perflubron exposure. Although a small increase in MCTR was noted, this was no greater than the increase seen with MARS. It may be that although the lowering of surface tension decreased mucociliary adhesive forces, the higher density and viscosity of the perflubron also decreased ciliary power through increased energy loss in the transfer of energy between the cilia and mucus, and that these effects balance each other out. It is also possible that because the MCTR was near maximal before the perflubron treatment, smaller increases in MCTR might not be statistically significant. In any event, it is unlikely that increased mucociliary clearance alone could account for the clinical observation of airway mucus obstruction, because if this were the case, mucus mobilization would principally occur immediately after the initiation of PLV.

There was an increase in mucin, but not lysozyme, secretion at all experimental time periods, although a smaller increase in lysozyme secretion may not have been detected because of a smaller number of samples evaluated. The increase in mucin secretion may lead to the production of a more viscous secretion that may be more likely to obstruct conducting airways.

There was a significant increase in tracheal mucin secretion, initially, at 40 min and again after evaporation following a 4-h immersion in perflubron. There was a greater increase in mucin secretion measured in the first set of experiments, an 86% increase in mucin secretion after 40 min and a 52% increase after 4 h plus evaporation, compared with the 30% increase at both times that we found in the second set of experiments to evaluate secretion pathways. This result seemed to be because of variability in the secretory response of individual animals.

The experiments allowing the perflubron to evaporate from the trachea after a 4-h immersion time were designed to mimic the clinical conditions that exist when PLV with perflubron is terminated and the fluorocarbon is allowed to evaporate from the airway surface during the switch to conventional mechanical ventilation. The data reported here are consistent with clinical reports that mucus accumulation has been observed at the induction and again at the end of PLV with perflubron. However, the increase in mucin secretion at both the start and the
end of the exposure periods suggests the possibility of different mechanisms causing mucus accumulation.

It is probable that perflubron initially mobilizes secretions by virtue of its surface-active properties and higher density combining to lift mucus off the epithelium. As the KHS is considered to be similar in composition to the normal periciliary fluid layer, this result may also be of clinical significance. After 4 h in a warmed shaking water bath and incubation in KHS alone, much of the endogenous mucus present on the epithelium would be slowly washed off the epithelial surface. This would explain the similar amounts of mucin measured in the KHS or perflubron bathing solutions at 4 h.

It is unlikely that perflubron directly induces secretion through receptor binding because the receptor-mediated effect, if present, should be short-lived. Nevertheless, perflubron could induce the release of mediators that secondarily trigger receptor-mediated secretion. This seems to be the mechanism responsible for the increase in mucus secretion, especially after the initial mobilization phase. Inhibition of the secretagogue effect by NDGA suggests that the hypersecretion is stimulated by arachidonic acid (AA) metabolites, probably leukotrienes generated through the lipoxygenase pathway, although products of the cyclooxygenase pathway, especially PGF$_{2\alpha}$, cannot be completely excluded by these experiments. Perflubron caused no histological or physiological damage to airway tissue, so AA activation appears not to be associated with inflammation. It has been shown that lipoxygenase pathway products are released on nasal provocation with cold, dry air, exposure to high altitude, and in exercise-induced asthma, in which this release may occur in part in response to physical stimuli such as changes in temperature or airway humidification. Leukotrienes are also released by physical stimuli including exposure to irritants. We suspect that AA metabolism may be induced by changes in the epithelial environment including changes in density, hydration, and tonic of the airway surface fluid.

The antioxidant NDGA is able to block directly and rapidly both the lipoxygenase and cyclooxygenase pathways, and NDGA suppressed mucin secretion both immediately (at 40 min) and after 4 h. Methylprednisolone only appeared to exert an effect after 4 h of incubation and did not show any effect at 40 min. Physiologically, methylprednisolone has a latent period for effects to be demonstrated, as this pathway of phospholipase A inhibition is mediated through lipocortin generation, which is time-dependent.

At the end of perflubron exposure, the bathing fluid around the tracheal segment was changed from KHS to perflubron. A change of microenvironment from liquid to air occurs because perflubron evaporates faster than KHS. This could stimulate secretion directly by mechanical changes in the epithelium or secondarily by stimulating AA metabolism.

After 4 h of perflubron plus evaporation, atropine and methylprednisolone partially inhibited, and NDGA and PIC completely inhibited, the increased mucin secretion. As expected, there was no inhibition of secretion with α- or β-adrenergic receptor antagonists. The partial inhibition by atropine suggests the involvement of cholinergic mediators activated by mechanical stimulation of the epithelium. The protease inhibitors were chosen to inhibit the kinin pathway and complement the system rather than to inhibit proteolytic secretagogues, such as elastase, because the healthy tracheal tissue of these animals should be relatively free of proteases, and components of the kinin pathway can be activated in response to physical stimuli. These data are also most consistent with a role for AA metabolites in producing the observed secretory effect.

Extrapolating these results to the clinical setting should be done carefully, as these studies were conducted in healthy ferret tracheal tissue, whereas PLV is used in patients with respiratory failure and often with airway inflammation. However, if the principal secretagogue effect is due to generation of AA metabolites, this effect might be even more pronounced in the inflamed airway.

It is unlikely that the generation of AA metabolites is the only cause of the increase in secretions noted during PLV, because locally generated metabolites of AA probably would be washed away during the course of PLV. A primary role for perflubron lifting and mobilizing mucus that is present on the airway surface is experimentally suggested by the greater initial increase (86%) in mucin noted at 40 min of exposure with less difference (18%) between perflubron and KHS after 4 h of incubation with no evaporation. Together, these data suggest that the mucus mobilization and secretion-modifying effect is not directly harmful and can be partially inhibited by phospholipase A inhibitors. Although these experiments were not designed to evaluate any potential therapeutic benefits of mucus mobilization or secretion by perflubron, the results lend some support to the speculation that perflubron might be beneficial for airway clearance in patients with profound airway obstruction due to impacted secretions.

**References**

12 Lundgren JD, Shellhammer JH, Kaliner MA. The role of eicosanoids in respiratory mucus hypersecretion. Ann Allergy 1985; 55:5–8