Effect of ultrasonically nebulized distilled water on airway epithelial cell swelling in guinea pigs

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Mochizuki, Hiroyuki, Yasushi Ohki, Hirokazu Arakawa, Kenichi Tokuyama, and Akihiro Morikawa. Effect of ultrasonically nebulized distilled water on airway epithelial cell swelling in guinea pigs. J. Appl. Physiol. 86(5): 1505–1512, 1999.—To investigate the pathogenesis of ultrasonically nebulized distilled water-induced airway narrowing, we studied the role of airway epithelial cells during a distilled water-inhalation challenge in an animal model of airway inflammation. Guinea pigs were divided into four groups: 1) a sham/saline (S/S) group: sham ozone followed by saline inhalation; 2) a sham/water (S/W) group: sham ozone followed by water inhalation; 3) an ozone/saline (O/S) group: ozone followed by saline inhalation; and 4) an ozone/water (O/W) group: ozone followed by water inhalation. After exposure to either 3.0 parts/million ozone or air at the same flow rate for 2 h, guinea pigs were anesthetized and tracheostomized, and then lung resistance (RL) was measured. For morphometric assessment, tissues were fixed with formaldehyde, stained with hematoxylin and eosin, and cut into transverse sections. Airway dimensions were either measured directly or calculated from the internal perimeter, the external perimeter, and airway wall area. There were no statistical differences in the values of RL before distilled water inhalation between the sham groups and the ozone groups. RL increased significantly after 10 min of distilled water inhalation in both the S/W group and the O/W group. In the S/W group, epithelial cells were swollen, and intercellular spaces were wider, resulting in significant increase in epithelial wall thickness, but there was no significant infiltration by inflammatory cells. In the O/S group, the epithelium showed infiltration by inflammatory cells without change in cell volume. In the O/W group, the epithelium showed both infiltration and a greater increase in epithelial wall thickness compared with the S/W group. These results suggest that airway epithelial cell swelling, induced by inhaled distilled water, increases with RL in guinea pigs and that this reaction may be accelerated by airway inflammation.

airway epithelial cell; experimental asthma; guinea pigs; ozone exposure; ultrasonically nebulized distilled water inhalation challenge

BRONCHOIAL HYPERRESPONSIVENESS (BHR) is defined as an exaggerated constrictive response of the airways to a wide variety of specific and nonspecific stimuli, with this phenomenon playing a central role in the pathophysiology of asthma (29, 31). Histamine- or methacholine-inhalation challenge are the most popular techniques to measure BHR, and a wealth of information has been accumulated about the clinical aspects of BHR. Ultrasonically nebulized distilled water-inhalation challenge is also used to measure BHR (2, 10).

However, distilled water-inhalation challenge does not correlate with histamine-inhalation challenge and is inhibited by inhaled furosemide, which does not affect histamine- or methacholine-inhalation challenges (3, 23). It has been suggested that inhaled distilled water does not appear to act directly on smooth muscles in airways and that changes in the osmolarity and ion composition of the periciliary fluid of airway epithelium are the most important factors in the airway narrowing induced by distilled water inhalation (1, 32). Inhaled distilled water may induce rapid ion and/or osmolar changes in airway fluid, which, in turn, may affect the activation of mast cells and other inflammatory cells or the stimulation of sensory nerve endings (5, 15), resulting in a distilled water-induced forced expiratory volume in 1 s (FEV₁) decrease in asthmatics. However, the precise mechanism of distilled water-induced airway narrowing is still unclear.

As shown by Fujimura et al. (12), the development of an animal model of distilled water-induced airway narrowing may be useful for studying the mechanisms of distilled water-inhalation challenge. One of the purposes of this study was to establish a reliable method to create a distilled water-responsive animal model. Previous reports have demonstrated an association between the development of BHR and inflammation after acute exposure to ozone (14). Among various animal models of bronchial asthma, the ozone-exposed model has been of particular interest, not only for BHR but also for epithelial injury and inflammatory cell infiltration into the bronchial wall (20, 27), and so we used ozone-exposed guinea pigs as an animal model for this report.

As to distilled water-induced airway narrowing, it has been reported that the mechanism of airway mast cell activation may not necessarily be involved (2, 33). It has been hypothesized that epithelial cell swelling induced by rapid ion transport across the epithelial cells, due to ion and/or osmolar changes in airway fluid during distilled water-inhalation challenge, results in decreasing FEV₁ in asthmatic patients (23, 33). Indeed, it has been demonstrated that the thickness of airway epithelial cells may change approximately two-fold (7) and that airway wall thickness significantly increases airway resistance (6). However, the effect of epithelial cell swelling during distilled water-inhalation challenge, which may be an important factor in increasing airway wall thickness, has not been studied. In this report, to investigate the pathogenesis of the distilled water-induced FEV₁ decrease, we developed an animal model of distilled water-induced airway...
narrowing using ozone exposure and we evaluated the epithelial cell swelling induced by inhaled distilled water.

**METHODS**

Study design. Hartley strain guinea pigs (males, 380–430 g each) were prepared for an assessment of airway responsiveness. The animals were divided into four groups (n = 6–9 in each group): 1) a sham/saline group: sham ozone subjects were placed in a chamber for 2 h, exposed to air at the same flow rate as for ozone, and were then subject to saline inhalation; 2) a sham/water group: sham ozone followed by water inhalation; 3) an ozone/saline group: ozone exposure followed by saline inhalation; and 4) an ozone/water group: ozone exposure followed by water inhalation. Four additional groups of guinea pigs (n = 6–9 in each group) were prepared for histological examination. All animals were killed, and their lungs and airways were excised for histological evaluation.

Ozone exposure. Guinea pigs inhaled 3.0 ± 0.8 (SD) parts/million of ozone for 2 h while awake and breathing spontaneously in an 18-liter exposure chamber described in previous reports (20, 27). Ozone was generated by passing 100% O2 through an ozone generator (model PZ-1, Kojima, Tokyo, Japan) regulated by a variable-voltage supply. The concentration of ozone was monitored continuously with an analyzer (Ozone Monitor EG-2001, Ebara Jitsugyo, Tokyo, Japan), with one side attached to a catheter inserted into the right pleural cavity and the other side attached to a catheter connected to a side port of the intratracheal cannula. Airflow was measured with a pneumotachograph (model TU-241T; Nihon Koden) connected to a transducer (model TP-6027; 5 cmH2O; Nihon Koden). All signals were recorded on a personal computer (using Mac Labo software), and lung resistance (RL) was calculated as described previously (34).

After stabilization of the baseline RL, all subjects inhaled aerosolized 0.9% saline with an ultrasonic nebulizer (Omron, NEU-07) for 1 min. After 15 min, distilled water was aerosolized for 1 min in the sham/saline group and the ozone/saline group, and saline was aerosolized for 1 min in the sham/saline group and the ozone/saline group. An ultrasonic nebulizer was connected near the animal’s side in a closed mechanical respiratory system. Thus subjects inhaled nebulized water with a tidal volume of 10 ml/kg and a frequency of 60 breaths/min. The mean effective nebulization air particles was 5.0 µm, and the total amount of nebulized solution inhaled in 1 min was 0.6 ml. RL and the mean systemic blood pressure were monitored immediately and at 1, 5, 10, 20, and 30 min after inhalation of nebulized distilled water.

Histological examination. To assess the morphological changes induced by inhalated nebulized distilled water and/or ozone exposure, animals in all groups were exsanguinated, and their lungs and tracheas were excised at 10 min after water or saline inhalation. A cannula was introduced into the proximal portion of the trachea, and the lungs were distended with 10% formaldehyde applied at a constant pressure of 25 cmH2O. Tissue specimens were taken from the trachea, main bronchi, and the lobar bronchi; embedded in paraffin; cut into 6-µm-thick sections; and stained with hematoxylin and eosin.

Airway dimensions. Airways that were cut transversely and did not show bifurcation or disruption of the wall were selected for measurement. Membranous and cartilaginous airway dimensions were measured with a digitizer (Fukuda Denshi, Cardio 500 system). The images were enlarged and traced, and the dimensions of the tracing were measured with a planimeter. Figure 1 shows the dimensions measured: the internal perimeter (Pı) and the internal area (Aı), defined by the lumen surface of the epithelium, and the external perimeter (Pı) and the external area (Aı), defined by the basement membrane (18). Where the epithelium was discontinuous, the internal radius of the adjacent portions...
of the epithelium. From the measured dimensions, the wall area ($A_w$) was calculated by subtracting the $A_i$ from the $A_e$ ($A_w = A_e - A_i$) (18).

The $A_e$ as a proportion of the “relaxed fully dilated” $A_e$ was calculated for each airway. Because $P_i$ is not altered by smooth muscle constriction or by lung inflation (17, 19) and because in the relaxed and dilated state $P_i$ is circular, the “relaxed and dilated” $A_i$ is given by $P_i^2/4\pi$, and the relaxed and dilated $A_e$ is given by $P_e^2/4\pi + A_w$.

The diameter of the maximally dilated airway was also calculated and is referred to as the calculated luminal diameter: the calculated diameter $= 2 \times \text{radius} = P_i/\pi$. The thickness of the $A_w$ is determined by subtracting the radius of a circle with a circumference equal to the $P_i$ ($R_o$) from the radius of a circle with a circumference equal to the perimeter of the area internal to the basement membrane ($R_d$).

Epithelial wall thickness ($T_w$) = $R_o - R_d = \sqrt{A_e/\pi} - \sqrt{A_i/\pi}$

Finally, $T_w$ was calculated from $P_i$. In this study, we also calculated $T_w$ from $P_e$ ($T_e$), and from $A_e$. We believe that $P_e$ does not change significantly during distilled water inhalation challenge and that $T_e$ could be useful, because some previous reports have suggested that inhaled distilled water does not induce direct or significant smooth muscle constriction (1, 32). We obtained the directly measured value of $T_w$, which is the mean of four points on the epithelium, but only in cartilaginous airways because $T_w$ is not constant in membranous airways. We also measured the areas of submucosa ($A_s$) and muscle ($A_m$), and we calculated the thicknesses of the submucosa ($T_s$) and muscle ($T_m$) using $P_i$ and $T_w$. We were unable to measure the $A_m$ of the cartilaginous group with airways of $>$1-mm diameter, because almost all of the airway muscles in this group showed significant deviation in the airway circle.

Radius of submucosa ($R_s$) = $\sqrt{P_i/2\pi + T_w}$

$T_s = \sqrt{(A_e/\pi + R_s^2)} - R_s$

Radius of muscle ($R_m$) = $\sqrt{P_i/2\pi + T_w + T_s}$

$T_m = \sqrt{(A_e/\pi + R_m^2)} - R_m$

The relative areas of the airway wall occupied by the epithelium were calculated in each different size group.

Relative wall area = $A_w/(A_w + A_e)$

Statistics. Data are reported as means $\pm$ SE. The nonparametric analysis of variance (Kruskal-Wallis method) was used to determine whether there was a significant difference between individual groups. If a difference was found, the Mann-Whitney U-test was used to evaluate the difference, with $P < 0.05$ considered significant. Data were analyzed with a computer using a standard statistical package (Statistics for Macintosh computers, StatSoft).

RESULTS

Changes in $R_L$. The baseline $R_L$ of the sham/saline and sham/water groups and the ozone/saline and ozone/water groups was $0.18 \pm 0.01$ and $0.21 \pm 0.01$ cmH$_2$O·ml$^{-1}$·s, respectively. There were no significant differences in baseline $R_L$ among these groups (Fig. 2).

In the sham/saline and ozone/saline groups, saline inhalation caused no statistical change in $R_L$ within 30 min. In contrast, distilled water inhalation caused a significant increase in $R_L$ after 10 min in the sham/water and ozone/water groups. Also, in the ozone/water group, distilled water inhalation caused a significant increase in $R_L$, with the maximal response at 10 min. Values of $R_L$ in the sham/water and ozone/water groups were significantly higher than those in the sham/saline and ozone/saline groups at each time measurement. The increase in $R_L$ induced by distilled water inhalation was significantly greater in the ozone/water group than in the sham/water group.

Changes in morphology. In this study, 34 guinea pigs were used for pathophysiological examination. Thirty-four sections of large airways and 32 sections of lung tissue that contained 116 bronchi and smaller airways were examined, and then 30 samples of large airways and 86 bronchi and smaller airways were chosen.

In the sham/ozone group, although no changes in epithelial cell volume or wall thickness were observed, 83% of the airway epithelium showed infiltration by inflammatory cells and partial occlusion of the lumen with mucus and cellular debris (Fig. 3C), compared with 7% in the sham/saline group (Fig. 3A). In the sham/water group, the epithelial cells, columnar ciliated cells, and goblet cells were swollen and intercellular spaces were wider, causing an increase in epithelial wall thickness (Fig. 3B). However, inflammatory cell infiltration was observed in only 13% of the samples, and the number of inflammatory cells involved was small. In the ozone/water group, 89% of the airway epithelium showed both infiltration by inflammatory cells and a significant increase in epithelial thickness; that is, epithelial cell swelling with widening of lateral intercellular spaces (Fig. 3D). Also, partial occlusion of the lumen with mucus and cellular debris was observed in 72% of the specimens.
Specimens were divided into four groups according to airway dimension calculated by $P_i$: small membranous (0.08–0.2 mm), large membranous (0.2–0.5 mm), small cartilaginous (0.3–1.0 mm), and large cartilaginous (1.0–1.7 mm). The frequency distributions of internal perimeter and external perimeter in each airway-sized group were not significantly different when the four groups were compared with one another, indicating that similar-sized airways were being examined. In each airway-sized group, the epithelial wall thickness of small airways was smaller than that of large airways. There was no difference in epithelial wall thickness between the sham/saline and ozone/saline groups in any airway-sized group, but epithelial wall thickness in the sham/water and ozone/water groups was an estimated twofold greater than in the sham/saline and ozone/saline groups in every airway-sized group (Table 1).

The absolute wall thickness of the cartilaginous airway epithelium in the sham/water and ozone/water groups was $\sim 1.4$- to 2-fold greater than that in the sham/saline and ozone/saline groups in all airway-sized groups (Table 1). In the cartilaginous bronchioles of $< 1$ mm in diameter, the mean values of the absolute epithelial wall thickness in the ozone/water group were 1.2-fold greater than those in the distilled water group. Also, the wall thickness calculated by internal perimeter was $\sim 1.5$- to 2.2-fold greater in the sham/water and ozone/water groups than in the sham/saline and ozone/saline groups in all airway-sized groups (Fig. 4.

**Fig. 3.** Light micrographs of airway epithelial cells in each group of guinea pigs. A, basement membrane; E, epithelium; L, lumen. A: sham/saline group. B: sham/water group. Epithelial cells were swollen, and intercellular spaces were wider, resulting in increase in epithelial wall thickness without infiltration by inflammatory cells. C: ozone/saline group. Epithelium in ozone group showed infiltration by inflammatory cells (arrows) without changes in cell volume or wall thickness. D: ozone/water group. Epithelium showed both infiltration by inflammatory cells (arrows) and a significant increase in epithelial wall thickness. Magnification $\times 400$ in A–D.
Table 1. Effect of ultrasonically nebulized distilled water inhalation on epithelial cell thickness in ozone-exposed guinea pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>Membranous &lt;0.2 mm</th>
<th>Membranous &gt;0.2 mm</th>
<th>Cartilaginous &lt;1.0 mm</th>
<th>Cartilaginous &gt;1.0 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham/saline</td>
<td>ND</td>
<td>ND</td>
<td>30.5 ± 2.7</td>
<td>37.5 ± 2.7</td>
</tr>
<tr>
<td>Sham/water</td>
<td>ND</td>
<td>ND</td>
<td>57.1 ± 6.0*</td>
<td>51.8 ± 3.4*</td>
</tr>
<tr>
<td>Ozone/saline</td>
<td>ND</td>
<td>ND</td>
<td>35.7 ± 1.9</td>
<td>36.8 ± 3.0</td>
</tr>
<tr>
<td>Ozone/water</td>
<td>ND</td>
<td>ND</td>
<td>65.4 ± 5.8†</td>
<td>56.6 ± 2.5†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sample nos. are 6–8 in each group. ND, not done.

DISCUSSION

It is well known that inhaled distilled water induces an FEV₁ decrease in asthmatic patients, although the mechanism of distilled water-induced airway narrowing is still unclear. One of the problems is that no reliable animal model of distilled water-inhalation challenge has been developed. Thus the development of such a model may be useful for studying the mechanisms of distilled water-inhalation challenge.

In this study, to establish a reliable technique for an inhaled distilled water-responsive animal model, we developed a guinea pig model of distilled water-induced airway narrowing using ozone exposure. Our model mimics clinical distilled water-inhalation challenge in children with asthma in some points: inhaled distilled water caused relatively slow changes in airways, and the distilled water-induced decrease in FEV₁ was slight compared with other inhalation challenges using some chemical mediators (23, 25). Using this model, we demonstrated that inhaled distilled water caused a significant increase in R₁, especially in animals exposed to ozone. Histologically, the thickness of the airway epithelium in both the sham/water and ozone/water groups showed a significant increase compared for airway obstruction, was greater in both the membranous and the cartilaginous airways of the distilled water-inhaled groups (Fig. 5). However, there was no difference in the wall area relative to the internal area between the ozone/saline group and the sham/saline group.

There were no significant differences in the submucosal thickness or the muscle thickness among the four groups in each airway size, except that the muscle thickness in sham/water group in the group of <0.2 mm in diameter was higher than that in other groups (Table 2). Also, there was no significant difference in the percent radius, which is the proportion of the thickness of submucosa or muscle to its radius, among the four groups in each airway size.

Table 1), and epithelial wall thickness calculated by internal perimeter in the water/saline group was a significant 1.3-fold greater than that in the sham/water group. The same tendency was observed in airway wall thickness calculated by using external diameter (∆), P < 0.01 compared with sham/saline group.

The wall area relative to the internal area within the basement membrane, which is one of the parameters for airway obstruction, was greater in both the membranous and the cartilaginous airways of the distilled water-inhaled groups (Fig. 5). However, there was no difference in the wall area relative to the internal area between the ozone/saline group and the sham/saline group.

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with the sham/saline group, with the ozone/water group increasing the greatest, whereas no changes in wall thickness was observed in the ozone/saline group.

Changes in epithelial wall thickness were observed in both distilled water-inhaling groups. It has been reported that inhaled distilled water does not appear to act directly on smooth muscles in airways and that the changes in osmolality and ion composition of the periciliary fluid of airway epithelium may be the most important factors in the bronchoconstriction induced by distilled water inhalation (24). That is, inhaled distilled water may induce rapid ionic and/or osmolar changes in airway fluid, which, in turn, may affect the activation of mast cells and other inflammatory cells or the stimulation of sensory nerve endings, resulting in bronchoconstriction (23).

Airway mast cell activation may not be involved in distilled water-induced airway narrowing. Verapamil, a known calcium antagonist expected to antagonize mast cell degranulation, was unable to inhibit distilled water-induced bronchoconstriction (2). Recently, we reported that inhaled furosemide protected against distilled water-induced airway narrowing, not only in atopic asthma but also in nonatopic asthma, indicating that this action may not be due to its stimulating action on airway mast cells (33).

The results of furosemide inhalation study are relevant to the mechanism of distilled water-induced airway narrowing. Furosemide, a loop diuretic agent, has been shown to inhibit the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter in the thick ascending loop of Henle (37), and it also inhibits the secretion of Cl\(^-\) into the bronchial lumen by blocking the cotransport of Na\(^+\) and Cl\(^-\) on the basolateral membrane of epithelial cells (30). It has been suggested that inhaled furosemide prevents bronchoconstriction induced by distilled water in asthmatic subjects (30), but it may not act directly at the airway smooth muscle level because of its lack of effect against both histamine- and methacholine-induced bronchoconstriction (28, 35).

We have suggested that rapid ion transport across epithelial cells by ionic and/or osmolar changes in airway fluid may play a role in the swelling of epithelial cells (23), resulting in the FEV\(_1\) decrease during distilled water-inhalation challenge in asthmatic patients. Indeed, epithelial cells are shrunk by raising the osmotic pressure and are swollen by decreasing the osmotic pressure (7). Inhaled distilled water induces a rapid decrease in the osmotic pressure in luminal fluid and water influx from luminal fluid to epithelial cells. Thus epithelial cells were swollen by influxed water, resulting in the reflux of ions from the serosal side across the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter.

This study evaluates the effect of epithelial cell swelling on distilled water-induced airway narrowing. After distilled water inhalation, the value of internal perimeter in the small cartilaginous groups in the sham/water group was 1.7 times higher, and in the ozone/water group 2.2 times higher, than in groups not subjected to distilled water inhalation. We calculated changes in airway resistance assuming laminar flow in a single airway completely surrounded by muscle, using the mean relative wall areas (26), and conventionally neglecting smooth muscle shortening, because we demonstrated only the effect of epithelial swelling on airway resistance in this study. That is, in membranous bronchi, the changes in airway resistance of the sham/water and ozone/water groups were 2.1 and 2.2 times greater, respectively, than in the sham/saline group. Also, in cartilaginous bronchi, the changes in airway resistance of the sham/water and ozone/water groups were 1.1 and 1.5 times greater, respectively, than in the sham/saline group.

Previous investigators have suggested that the same degree of muscle shortening causes greater airway narrowing in airways with thick walls (4, 11). The relationship between airway area and changes in airway resistance that occur as muscle shortens has been described by Moreno et al. (26). Clinically, in some asthmatic patients, especially in moderate or severe
types of asthma, the value of FEV\textsubscript{1} is usually low, suggesting the presence of chronic airway muscle shortening. Although increased wall thickness has been described as a feature of asthma (9, 13), there is little evidence for abnormal smooth muscle function in hyperresponsive airways (8, 36). The chronic inflammatory process present in the airway wall in patients with asthma is associated with cellular infiltration, deposition of connective tissue, hypertropy of smooth muscle, goblet cell metaplasia of the epithelium, and an inflammatory exudate containing mucosa in the airway lumen (9, 13). Thus the data reported here suggest the possibility that distilled water inhalation may lead to excessive airway narrowing with epithelial cell swelling during airway inflammation in asthmatics.

Furthermore, epithelial cell swelling may be accelerated by these inflammatory reactions in and under the airway epithelium, because most chemical mediators induced by inflammatory reactions stimulate the production of secondary messengers, which activate ion channels in airway epithelial cells (16, 22).

An increase in epithelial wall thickness during distilled water inhalation was significant in cartilaginous bronchioles of <1 mm in diameter in the ozone/water group. This increase in epithelial wall thickness may be the cause of the statistical difference in RL between the sham/water group and the ozone/water group. A positive explanation for the prominent increase in epithelial wall thickness in the lobar bronchi after ozone exposure is the combination of the effect of baseline epithelial wall thickness and the airway size-dependent effect of ozone on epithelial cell swelling. The epithelial thickness and quantity of epithelial cells in large airways is greater than in smaller airways (21). On the other hand, deposition of nebulized distilled water may be low in large airways, trachea, and main bronchi. Furthermore, Lum et al. (21) suggested that ozone-induced histological change, which is most prominent in the terminal bronchioles and smaller airways, may increase susceptibility to damage caused by ozone. The direct effect of ozone on epithelial cell swelling was not demonstrated in a comparison with the sham/saline in our data. Taken together, the total increase in epithelial cell swelling induced by distilled water inhalation may be greatest in lobar bronchi, especially in the ozone/water group.

Consequently, our results suggest that epithelial wall swelling is one of the mechanisms underlying distilled water-induced airway narrowing and that narrowing may be accelerated by airway inflammation. This suggestion is compatible with previous clinical studies showing that the distilled water-induced FEV\textsubscript{1} decrease in asthmatic subjects is related to osmotic changes in airways (2, 23). However, we could not measure osmolar changes directly in airway fluid during provocation tests on guinea pigs. Further investigations are needed to clarify the common mechanisms involved in distilled water-induced airway narrowing in asthma. We believe that our model may be helpful for investigating the mechanism of distilled water-induced airway narrowing in asthmatic subjects.

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