Effect of inhaled indomethacin on distilled water-induced airway epithelial cell swelling

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Mochizuki, Hiroyuki, Yasushi Ohki, Hirokazu Arawaka, Masahiko Kato, Kenichi Tokuyama, and Akihiro Morikawa. Effect of inhaled indomethacin on distilled water-induced airway epithelial cell swelling. J Appl Physiol 92: 155–161, 2002.—We evaluated the mechanism of the antiasthmatic effect of inhaled indomethacin (Indo) by using an animal model (guinea pigs) of airway inflammation. After being exposed to either ozone or room air at identical flow rates (5 l/min) for 2 h, guinea pigs were anesthetized, tracheostomized, and lung resistance (RL) was subsequently measured. Guineapigs inhaled either saline or Indo (1.5 mg/ml) for 1 min before undergoing an ultrasonically nebulized distilled water (UNDW) inhalation test. RL increased significantly after 10 min of UNDW inhalation in the room air and ozone groups but more so in the ozone group. This increase in RL was significantly suppressed by pretreatment with Indo. In the morphometric assessment of airway mucosa, a significant swelling of the epithelial cells after UNDW inhalation was observed in both the room air and ozone groups but especially so in the ozone group. This increase was also suppressed with Indo pretreatment. These results suggest that the increase in RL and the swelling of airway epithelial cells induced by inhaled UNDW in ozone-exposed guinea pigs was suppressed by pretreatment of inhaled Indo and that this suppression may be one of the reasons for the antiasthmatic effect of inhaled Indo.

Airway epithelial cell; guinea pigs; ozone exposure; ultrasonically nebulized distilled water inhalation challenge

INHALED ULTRASONICALLY NEBULIZED DISTILLED WATER (UNDW) induces a decrease in forced expiratory volume in 1 s (FEV1) in asthmatic patients (3, 15, 39). Inhaled indomethacin (Indo), which is a potent suppressor of cyclooxygenase and an inhibitor of prostaglandin synthesis, has been reported to inhibit UNDW-induced FEV1 decrease (8, 37).

It has been suggested that inhaled UNDW does not act directly on smooth muscle in airways and that changes in the osmolarity and ion composition of the periciliary fluid of airway epithelia are the most important factors in airway narrowing induced by UNDW inhalation (2, 36). According to previous reports (9, 19), inhaled UNDW induces rapid ionic and/or osmolar changes in the 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 a distilled water-induced FEV1 decrease in asthmatic patients. However, the precise mechanism is still unclear.

We have previously developed an ozone-exposure animal model, in which UNDW-induced bronchoconstriction and histological changes on airway epithelial cells were able to be evaluated (28), and suggested that one of the mechanisms of the UNDW-induced FEV1 decrease in asthmatic patients may be dependent on volume changes in epithelial cells, that is, cell swelling (30–31). It has been demonstrated that the thickness of airway epithelial cells is able to change approximately twofold (11) and that airway wall thickness significantly increases airway resistance (10).

Previous studies with asthmatic patients have reported benefits achieved by using inhaled furosemide, a Na⁺-K⁺-Cl⁻ cotransporter inhibitor, to reduce airway hyperresponsiveness to exercise (7) or to UNDW (35). There are similarities between the beneficial effects of Indo and furosemide: both inhibit ion transport across airway epithelia (27) and attenuate asthmatic responses against UNDW (37). It has been suggested that epithelial cells are shrunk by raising the osmotic pressure and are swollen by decreasing the osmotic pressure (11) and that rapid ion transport across epithelial cells by ionic and/or osmolar changes in the airway fluid may play a role in the swelling of epithelial cells (29). Indo also suppresses synthesis of prostaglandins, which induce the activation of ion channels in airway epithelial cells (1).

Therefore, we hypothesized that inhaled Indo prevents UNDW-induced broncho-obstruction by acting as a suppressor of epithelial cell swelling through inhibition of ion channel activity and/or prostaglandin synthesis. To investigate the pathogenesis of the inhaled Indo-induced antiasthmatic effect, we evaluated the effect of inhaled Indo on epithelial cell swelling induced by inhaled UNDW by using ozone-exposed guinea pigs.

MATERIALS AND METHODS

Study design. Hartley-strain guinea pigs (male, 385–440 g) were prepared for an assessment of airway responsiveness.

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**Table 1. Study design**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Exposure</th>
<th>Pretreatment</th>
<th>Provocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Room air</td>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>2</td>
<td>Room air</td>
<td>Saline</td>
<td>Distilled water</td>
</tr>
<tr>
<td>3</td>
<td>Ozone</td>
<td>Saline</td>
<td>Distilled water</td>
</tr>
<tr>
<td>4</td>
<td>Room air</td>
<td>Indo</td>
<td>Distilled water</td>
</tr>
<tr>
<td>5</td>
<td>Ozone</td>
<td>Indo</td>
<td>Distilled water</td>
</tr>
</tbody>
</table>

Indo, indomethacin.

We divided the animals without pretreatment with Indo inhalation into three groups as follows (Table 1): 1) room air/saline group (n = 8); animals were placed in a chamber for 2 h and exposed to room air at the same flow rate as the ozone group, followed by administration of nebulized saline; 2) room air/water group (n = 8); room air followed by nebulized water inhalation; and 3) ozone/water group (n = 8); ozone exposure followed by nebulized water inhalation.

Animals pretreated with Indo inhalation were divided into two groups: 1) room air/water+Indo group (n = 6) and 2) ozone/water+Indo group (n = 6). Part of the data of the groups, a room air/saline group and a room air/water group, has already been reported (28).

Five additional groups of guinea pigs 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 ppm (mean ± SD) of ozone or room air for 2 h (flow rate = 5 l/min) while awake and breathing spontaneously in an 18-liter exposure chamber of a type described in previous reports (25, 34). Ozone was generated by passing 100% O₂ through an ozone generator (model PZ-1, Kojima, Tokyo) regulated by a variable-voltage supply. The concentration of ozone was monitored by using an analyzer (ozone monitor EG-2001, Ebara Jitsugyo, Tokyo), which checks the ozone concentration continuously every minute. Room temperature was 20–25°C.

**Assessment of airway hyperresponsiveness to distilled water.** Airway responsiveness to inhaled distilled water was assessed after ozone exposure. Guinea pigs were anesthetized with pentobarbital (1 mg/kg iv). A tracheal cannula (10 mm in length with an inner diameter of 2.7 mm) was inserted into the lumen of the cervical trachea by using tractechometry and secured with a suture. A polyethylene catheter was inserted into the left carotid artery to monitor blood pressure with a pressure transducer. The right external jugular vein was cannulated for the administration of suxamethonium (5 mg/kg iv) 10 min before the testing to stop spontaneous breathing.

Guinea pigs were placed in a supine position with the intratracheal cannula connected to a constant-volume mechanical ventilator (model SN-480–7, Shinano, Tokyo). A tidal volume of 10 ml/kg and a frequency of 60 breaths/min were used. Transairway pressure was measured with a pressure transducer (50 cmH₂O; model TP-603T, Nihon Koden, Tokyo), 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 (5 cmH₂O; model TP-602T, Nihon Koden). All signals were recorded on a personal computer (using Mac Labo software), and lung resistance (Rl) was calculated as described previously (4, 28).

After stabilization of baseline RL, all animals inhaled aerosolized 0.9% saline or Indo solution with an ultrasonic nebulizer (NEU-07, Omron) for 1 min. Indo solution (3 mg/ml in distilled water containing sodium bicarbonate; Sumitomo Pharmaceutical, Tokyo) was diluted with 0.9% saline to 1.5 mg/ml. After 15 min, distilled water was aerosolized for 1 min in the water inhalation groups, and saline was aerosolized for 1 min in the saline inhalation groups. An ultrasonic nebulizer was connected near the animal’s side in a closed mechanical respiratory system. Thus animals inhaled nebulized water with a tidal volume of 10 ml/kg and a frequency of 60 breaths/min. Mean diameter of nebulized water particles was 5.0 μm, and the total amount of nebulized solution inhaled in 1 min was 0.6 ml. RL and 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 inhaled nebulized distilled water and/or ozone exposure, animals in five 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 distented with 10% formalin applied at a constant pressure of 25 cmH₂O. Tissue specimens were taken from the trachea, main bronchi, and lobar bronchi, embedded in paraffin, cut into 6-μm-thick sections, and stained with hematoxylin and eosin.

We measured the number of polymorphonuclear leukocytes (PMNs) in the epithelium to quantify the degree of airway inflammation induced by ozone and/or distilled water. **Airway dimensions.** Airways that were cut transversely and did not show bifurcation or disruption of the wall were selected for measurement. Images were enlarged and traced, and the dimensions of the tracing were measured with a curvimeter. Membranous and cartilaginous airway areas were measured with a digitizer (CARITIO 500 system, Fukuda Denshi). Figure 1 shows the dimensions measured:

- Internal perimeter (Pᵢ) and internal area (Aᵢ), defined by the lumen surface of the epithelium, and external area (Aₑ),
where $T$ and because in the relaxed and dilated state by smooth muscle constriction or by lung inflation (22, 24) for 1 min, the trachea was dissected from the guinea pigs (5).

In a small plastic well on ice, we removed epithelial cells from the trachea with the edge of a glass slide (5), collected them with 0.5 ml ice-cold phosphate-buffered saline, and immediately sonicated them for 30 s at 4°C with a sonicator (Ohtake Works, Tokyo). After sonication, the homogenate was centrifuged at 12,000 rpm for 5 min to remove cells and large fragments. The final supernatant was frozen and stored at −80°C for later use. We measured prostaglandin E2 using a commercial kit (R&D Systems, Minneapolis, MN). We measured protein concentration in each sample using a Bio-Rad protein assay (Bio-Rad Laboratories).

**Statistics.** Data are conventionally reported as means ± SE. Nonparametric ANOVA (Kruskal-Wallis method) was used to determine whether there was a significant difference between individual groups ($P < 0.05$). If a significant overall difference was found, a Mann-Whitney $U$-test was performed to assess the significant difference between individual groups, with $P < 0.05$ considered significant. Assessing the time course of $R_l$, we used the Bonferroni test for analysis of repeated measures across time and compared $R_l$ values between baseline and postinhalation. Data were analyzed with a computer using standard statistical packages.

**RESULTS**

**Changes in $R_l$.** Baseline $R_l$ of the room air/saline group, the room air/water group, and the ozone/water groups were $0.19 ± 0.02$, $0.17 ± 0.03$, and $0.20 ± 0.02$ cmH$_2$O·ml$^{-1}$·s$^{-1}$, respectively. Baseline $R_l$ of the room air/water+Indo group and the ozone/water+Indo group were $0.21 ± 0.01$ and $0.23 ± 0.03$ cmH$_2$O·ml$^{-1}$·s$^{-1}$, respectively. There were no significant differences in baseline $R_l$ among these groups.

In the room air/saline group, saline inhalation caused no statistical change in $R_l$ within 30 min (data not shown). In contrast, distilled water inhalation caused a significant increase in $R_l$ after 10 min in the room air/water group and especially so in the ozone/water group, which had a maximal response at 10 min. Values of $R_l$ in the room air/water and ozone/water groups were significantly higher than those in the room air/saline group at each time measurement. However, the increase in $R_l$ induced by ozone plus distilled water inhalation was significantly suppressed in the ozone/water+Indo group (Fig. 2).

**Changes in morphology.** In this study, 41 guinea pigs were used for pathophysiological examination. We examined 41 sections of large airways and 40 sections of lung tissue, which contained 143 bronchi and smaller

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**Table 2. Effect of inhaled Indo on ultrasonically nebulized distilled water-induced epithelial cell thickness in ozone-exposed guinea pigs**

<table>
<thead>
<tr>
<th></th>
<th>No. of PMNs, per mm$^3$</th>
<th>Membranous Bronchioles, μm</th>
<th>Cartilaginous Bronchioles, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤1.0 mm</td>
<td>&gt;1.0 mm</td>
</tr>
<tr>
<td>Room air/saline</td>
<td>266 ± 59(8)</td>
<td>9.5 ± 1.3(9)</td>
<td>15.9 ± 2.2(7)</td>
</tr>
<tr>
<td>Room air/water</td>
<td>575 ± 94*(8)</td>
<td>16.2 ± 3.4*(8)</td>
<td>35.4 ± 4.4*(8)</td>
</tr>
<tr>
<td>Ozone/water</td>
<td>1998 ± 270*(8)</td>
<td>21.2 ± 3.2*(7)</td>
<td>34.5 ± 5.2*(6)</td>
</tr>
<tr>
<td>Room air/water+Indo</td>
<td>994 ± 105*(6)</td>
<td>19.2 ± 3.2*(8)</td>
<td>29.2 ± 4.2(6)</td>
</tr>
<tr>
<td>Ozone/water+Indo</td>
<td>903 ± 207*(6)</td>
<td>14.5 ± 3.6(8)</td>
<td>29.3 ± 5.5(6)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses are the $n$ values of each sample. Epithelial cell thickness was calculated by inner perimeters. Polymorphonuclear leukocytes (PMN) numbers were calculated in samples of the cartilaginous group (>1.0 mm). *$P < 0.05$. Each $P$ value was obtained from comparisons between the value of the room air/saline group and the other groups.
airways, and then chose 36 samples of large airways and 74 bronchi and smaller airways.

The number of PMNs in the epithelia in each group is shown in Table 2. PMN numbers in the ozone/saline and ozone/water groups were significantly higher than in the room air/saline group, and the numbers in the ozone/saline + Indo and ozone/water + Indo groups were higher than in the room air/saline group. However, the number of PMNs in the epithelium in the ozone/water group was significantly higher than in the two Indo-pretreated groups ($P < 0.05$ and $P < 0.01$, respectively). These results indicate that airway inflammation was induced by ozone inhalation in our study and that Indo could suppress part of the combined effect of ozone and water.

There was no morphological difference in the epithelium between the intact animal and the room air/saline group (Fig. 3, A and B, respectively). In the room air/water group, epithelial cells, columnar ciliated cells, and goblet cells were swollen and intercellular spaces were wider, thereby causing an increase in epithelial wall thickness (Fig. 3C). In the ozone/water

Fig. 3. Light micrographs of airway epithelial cells in each group of guinea pigs. A: intact animal. B: room air/saline group. C: room air/water group. D: ozone/water group. The epithelium showed both infiltration by inflammatory cells and a significant increase in epithelial wall thickness. E: ozone/water + Indo group. The epithelium showed slight infiltration by inflammatory cells, some intercellular vacuoles, but no increase in wall thickness. Bar represents 20 $\mu$m. Magnification in A–E was $\times 400$. 

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group, airway epithelium showed both infiltration by inflammatory cells and a significant increase in thickness, that is, epithelial cell swelling with widening of lateral intercellular spaces (Fig. 3D). In the ozone/water + Indo group, the airway epithelium was not significantly thicker, and the number of infiltrated inflammatory cells was small (Fig. 3E).

Frequency distributions of \( P_i \) within each airway-sized group were not significantly different, indicating that similar-sized airways had been selected. In each airway-sized group, the epithelial wall thickness of small airways was smaller than that of large airways (Table 2).

Wall thicknesses were 1.5–2.2 times greater in the room air/water and ozone/water groups than in the room air/saline group in all of the airway-sized groups (Table 2), and epithelial wall thickness in the ozone/water group was significantly larger than the room air/water group and the room air/saline group or between the ozone/water + Indo group and the room air/saline group in all of the airway-sized groups.

Measurement of prostaglandin E\( _2 \) in epithelial cells

The concentration of prostaglandin E\( _2 \) in the control group, which inhaled saline, was greater than concentrations in the Indo-inhalation groups (Table 3). In particular, the \( P \) value in the group of 1.5 mg/ml (4.2 \( \times \) 10\( ^{-3} \) M) Indo inhalation was significantly lower than in the control group (\( P < 0.01 \)). These data suggest that 1.5 mg/ml inhaled Indo was enough to act as a cyclooxygenase inhibitor on airway epithelial cells in guinea pigs.

DISCUSSION

It has been reported that inhaled UNDW induces a decrease in FEV\( _1 \) in asthmatic patients (8, 14, 38) and that inhaled Indo inhibits this decrease (37). Bianco et al. (8) suggested that an anti-inflammatory action due to the inhibition of local prostaglandin synthesis in the airways is a possible mechanism of this reaction, but the mechanism responsible for the antiasthmatic activity of inhaled Indo is not well understood.

As shown by Fujimura et al. (17), the development of an animal model of distilled water-induced airway narrowing may be useful for studying the mechanisms of distilled water inhalation-induced airway narrowing. Previously, to establish a reliable technique for an inhaled-UNDW animal model, we developed a guinea pig model of UNDW-induced airway narrowing by using ozone exposure (28). Changes in RL in our model suggest airway obstruction consistent with the airway obstruction found in studies using children and assessed by using spirometry or FEV\( _1 \).

In this report, by using this model, we demonstrated that inhaled UNDW caused an increase in RL, especially in animals exposed to ozone, and that this increase was significantly suppressed by Indo pretreatment. Also, in a histological study, the thickness of airway epithelium in both the room air/water and ozone/water groups showed a significant increase (with the ozone/water group increasing the greatest) compared with the room air/saline group, whereas no changes in wall thickness were observed in the room air/water + Indo and ozone/water + Indo groups. The number of PMNs in the epithelium in the ozone/water group was significantly higher than in the two Indo-pretreated groups, and these results indicate that airway inflammation was induced by ozone inhalation. Although the number of PMNs in the epithelium in the ozone/saline + Indo and ozone/water + Indo groups were higher than in the room air/saline group, there was no significant difference between the number of PMNs in the epithelium in the ozone/saline + Indo and the saline/water groups. We speculated that the Indo solution has a slight effect, similar to inhaled distilled water, on the accumulation of PMNs in the airway epithelium.

It has been reported that inhaled UNDW does not appear to act directly on smooth muscle in airways and that changes in osmolarity and ion composition of the periciliary fluid of airway epithelium may be the most important factors in airway narrowing induced by distilled water inhalation (18, 31). That is, inhaled distilled water may induce rapid ionic and/or osmolar changes in airway fluid, which in turn may affect the stimulation of mast cells and other inflammatory cells or the stimulation of sensory nerve endings, resulting in bronchoconstriction (29).

However, airway mast cell activation may not be involved in distilled water-induced airway narrowing. Verapamil, a known Ca\( ^{2+} \)-channel blocker expected to inhibit mast cell degranulation, was unable to inhibit distilled water-induced bronchoconstriction (3). Previously, 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 is not due to its stimulating action on airway mast cells (38).

Results of our Indo inhalation study may be relevant to the mechanism of distilled water-induced airway narrowing. Indo has been shown to inhibit the Cl\( ^{-} \) channel on the mucosal membrane of airway epithelial cells (27) and to inhibit the secretion of Cl\( ^{-} \) into the bronchial lumen (40). 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

Table 3. Effect of inhaled Indo on prostaglandin synthesis in epithelial cells in guinea pigs

<table>
<thead>
<tr>
<th>Indo concentration (mg/ml)</th>
<th>PGE( _2 )/Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>206.5 ± 60.0</td>
</tr>
<tr>
<td>Indo 0.3 mg/ml</td>
<td>131.9 ± 33.7</td>
</tr>
<tr>
<td>Indo 1.5 mg/ml</td>
<td>64.9 ± 4.6*</td>
</tr>
<tr>
<td>Indo 7.5 mg/ml</td>
<td>44.6 ± 18.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE (\( n = 6 \)). PGE\( _2 \)/protein, prostaglandin E\( _2 \) (pg/ml)/protein concentration (mg/ml). * \( P < 0.05 \). Each \( P \) value was obtained from the comparison between the value of the saline-inhalation group and the other groups.
of epithelial cells (30). Indeed, epithelial cells are shrunk by raising osmotic pressure and are swollen by decreasing osmotic pressure (11). Inhaled UNDW induces a rapid decrease in the surrounding osmotic pressure in luminal fluid and water influx from luminal fluid into epithelial cells, resulting in the swelling of cells. Thus inhaled Indo may suppress epithelial cell swelling by inhibiting Cl\(^-\) channel activity, thereby preventing the UNDW-induced airway narrowing.

After distilled water inhalation, epithelial cell wall thickness in the small cartilaginous groups in the room air/water group was 1.7 times higher and in the ozone/water group was 2.1 times greater and 2.2 times greater, respectively, than in the ozone/saline group. Also, in cartilaginous bronchi, changes in airway resistance of ozone/water groups was 1.5 times greater than in the room air/saline group (28).

Previous investigators (6, 16) suggested that the same degree of muscle shortening causes greater airway narrowing in airways with thick walls. The chronic inflammatory process present in the airway wall in patients with asthma is associated with cellular infiltration, deposition of connective tissue, hypertrophy of smooth muscle, goblet cell metaplasia of the epithelium, and an inflammatory exudate containing mucus in the airway lumen (13, 20). Also, other data suggest that distilled water inhalation may lead to excessive airway narrowing with epithelial cell swelling during airway inflammation in asthmatics (28). Thus it is possible to suppress the epithelial cell swelling-induced airway narrowing by pretreatment with Indo inhalation.

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 (21, 26). In this report, inhaled Indo (1.5 mg/ml) successfully suppressed prostaglandin synthesis in epithelial cells. Indo exerts its inhibitory effect on local prostaglandin synthesis in the airways. In addition to this, Coleridge et al. (12) reported that prostaglandins can stimulate lung irritant receptors and afferent C-fibers. Inhaled Indo may therefore modify UNDW-induced broncho-obstruction by inhibiting prostaglandin synthesis in the airways, which would otherwise induce epithelial cell swelling and nervous system stimulation.

Consequently, our results suggest that epithelial cell swelling, which is one of the mechanisms underlying distilled water-induced airway narrowing, may be suppressed by inhaled Indo. We speculated that inhaled Indo suppresses epithelial cell swelling by inhibiting ion channel activity and/or the prostaglandin synthesis in airway epithelial cells. However, further investigations are needed to clarify the precise mechanism of Indo on distilled water-induced airway narrowing in asthmatics.

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REFERENCES


