Hyperventilation-induced airway injury and vascular leakage in dogs: effects of $\alpha_1$-adrenergic agonists

ARTHUR N. FREED, VARSHA TASKAR, BRIAN SCHOFIELD, AND CHIHARU O Mori

Hyperventilation-induced airway injury and vascular leakage in dogs: effects of $\alpha_1$-adrenergic agonists. J. Appl. Physiol. 83(6): 1884–1889, 1997.—Adrenergic agonists inhibit hyperventilation-induced bronchoconstriction (HIB) in dogs. We tested the hypothesis that $\alpha_1$-agonists inhibit HIB by reducing bronchovascular leakage and edema that theoretically could cause airway obstruction. Peripheral airways were isolated by using a bronchoscope; pretreated with either methoxamine (Mx), norepinephrine (NE), or saline aerosol; and then exposed to a 2,000 ml/min dry-air challenge (DAC) for 2 min. Colloidal carbon was injected before DAC and used to quantify bronchovascular permeability. Mx-, NE-, and vehicle-treated airways were prepared for morphometric analysis within 1 h after DAC. Light microscopy revealed that the 2-min DAC produced minimal bronchovascular leakage and little epithelial damage. However, pretreatment with either Mx or NE significantly enhanced dry air-induced bronchovascular hyperpermeability and mucosal injury. The increased damage associated with these $\alpha_1$-agonists implicates a protective role for the bronchial circulation. The fact that $\alpha_1$-agonists inhibit HIB suggests that neither dry air-induced leakage nor injury directly contributes to the development of airway obstruction. In addition, our data suggest that $\alpha_1$-agonists attenuate HIB in part by augmenting hyperventilation-induced bronchovascular leakage and by replacing airway water lost during a DAC.

bronchovascular permeability; exercise-induced asthma; goblet cells; hyperventilation-induced bronchoconstriction; mast cells

HYPERVENTILATION-INDUCED bronchoconstriction (HIB) in canine peripheral airways resembles in many respects hyperpnea- or exercise-induced asthma in human subjects. The time course over which HIB develops and subsides and the responses to variations in stimulus strength and duration are remarkably similar (11, 17). HIB is also associated with airway epithelial cell damage (14, 15, 31) and the release of biochemical mediators in dogs (15, 24, 31) and asthmatic humans (9, 22, 30) alike.

Hyperventilation with dry air increases evaporative water loss. Subsequent increases in local airway fluid osmolarity may then trigger mast cell degranulation and airway smooth muscle constriction (1). Studies of asthmatic subjects provide indirect evidence suggesting that $\alpha_1$-agonists diminish either hyperpnea-induced microvascular hyperperfusion, engorgement, bronchovascular leakage, or edema formation (8, 18). However, there remains a dearth of data demonstrating that any of these events actually contribute to the development of HIB in asthmatic patients. Hyperventilation with dry air does increase bronchovascular blood flow (2) and permeability (14) in dogs and may cause airway narrowing via bronchovascular congestion, leakage, and the formation of edema. Theoretically, $\alpha_1$-adrenergic agents can protect against HIB by inhibiting the development of these phenomena.

In the present study, we tested the hypothesis that $\alpha_1$-adrenergic agonists inhibit HIB by reducing bronchovascular hyperpermeability. Methoxamine (Mx) and norepinephrine (NE) were selected for use in this study because each has been shown to be effective in inhibiting hyperpnea-induced airway obstruction in human asthmatic subjects (8, 18) and in canine peripheral airways (25). However, we found that both drugs enhance bronchovascular permeability, indicating that airway microvascular leakage is unlikely to play a pivotal role in the development of HIB.

METHODS

Experimental Techniques

Dogs were handled and maintained in accordance with the standards set forth in the Policy and Procedures Manual published by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine and Public Health.

Peripheral airway preparation. Male mongrel dogs [18 ± 0.9 (mean ± SE) kg, n = 9] were anesthetized with pentobarbital sodium (30 mg/kg/iv), supplemented with pentobarbital sodium (30 mg/iv) as needed. Depth of anesthesia was assessed by canthal reflex, heart rate (HR), blood pressure, and the presence of spontaneous movement or breathing. After tracheotomy and insertion of a twin portal tracheal tube into the wedged sublobar segment; the other lumen was used to deliver compressed, dry 5% CO2 air at room temperature at a rate of 200 ml/min into the wedged sublobar segment; the other lumen was connected to a pressure transducer (Statham; Gould, Oxnard, CA) and was used to measure airway pressure at the tip of the bronchoscope (Pb).

Dry-air challenge (DAC). After a stable baseline Pb was established, insufflation of dry 5% CO2 in air was increased
from 200 to 2,000 ml/min for 2 min. At the end of 2 min, it was reduced to the baseline flow rate of 200 ml/min.

Aerosol administration of Mx and NE. Either Mx (Sigma, St. Louis, MO; 40 mg/ml in saline), NE (Sigma; 0.5 mg/ml in saline), or saline (0.9%) aerosols were generated by using an ultrasonic nebulizer (Ultra-Neb 100; DeVilbiss, Somerset, PA) that delivered ~14 µl/min. The catheter was temporarily removed, then the aerosol was generated in air with 5% CO2 and was delivered for 2 min through the suction port of the bronchoscope and into the wedged segment at 200 ml/min.

DAC was done immediately after reestablishing a stable baseline Pb.

Tissue removal and preparation. After the animal was exsanguinated, a median sternotomy was performed, the pulmonary artery and left atrium were cannulated, and the pulmonary vasculature was perfused with Hanks’ buffered salt solution. Both lungs were removed within 1 h after DAC was completed, and they were prepared for morphometric analysis. After each lobe was cannulated, Streck tissue fixative (Streck Laboratories, Omaha, NE) was instilled to an inflation pressure of 20 cmH2O. Lobes were then immersed in fixative for 24–48 h before dissection. After fixation, the wedge site in each lobe was determined via an airway map that was constructed at the beginning of the experiment, and sublobar bronchi were dissected free from the parenchyma. The bronchial tree was photocopied, diagrammed, and cut serially into ~3-mm-long rings and labeled for image analysis. The bronchial rings were dehydrated via a continuous ethanol series and were embedded in glycolmethacrylate by using a JB-4 embedding kit (Polysciences, Warrington, PA). One 2- to 3-µm cross section of each airway ring was stained with periodic acid-Schiff (PAS), and one was stained with toluidine blue (TB) and naphthol yellow S.

Morphometric analysis. Airways with cross sections ranging from 0.5- to 4.4-mm diameter were examined by using light microscopy and an image-analysis system (Sigma Scan; Jandel Scientific, Corte Madera, CA). Airways ≥4.5 mm in diameter were excluded because of their proximity to the bronchoscope (~5-mm diameter). Control airways were obtained from unwedged sublobar segments that were not exposed to DAC but were located adjacent to a challenged sublobar segment. All airway cross sections were categorized as either bronchi (with cartilage) or bronchioles (without cartilage). The relative condition of the airway mucosa was assigned to one of three categories for analysis: normal mucosa containing ciliated and goblet cells (C + G), normal mucosa containing primarily ciliated cells (C – G), and damaged mucosa composed of either nonciliated or denuded airway surfaces (14). The perimeter of the basement membrane (PBM) and the lengths of perimeter containing C + G, C – G, and damaged mucosa were measured. PBM was used to calculate the maximally relaxed bronchial diameter (D = PBM/π). Four to six cross sections were evaluated from each experimental sublobar segment, and two airway cross sections were examined from each control segment. Measurements were made on each airway cross section at a magnification of ×400, and the overall average per sublobar segment was calculated for each treatment. Ciliated cells per millimeter and goblet cells per millimeter of basement membrane were counted in C + G and C – G mucosa of PAS-stained tissues, and goblet-to-ciliated cell (G/C) ratios were calculated. Goblet cells were identified by their shape and affinity for PAS. The numbers of mast cells in the lamina propria and submucosa of the bronchial wall located directly below either normal or damaged mucosa were counted in cross sections stained with TB. Mast cells were identified as cells that had granules stained with TB. Mast cell density was expressed as number per square millimeter of lamina propria or submucosa located below either normal or damaged mucosa. The area of colloidal carbon in the vascular wall of vessels located under either normal ciliated or damaged mucosa, expressed as square micrometers per square millimeter of airway tissue, was used to quantify microvascular leakage in the lamina propria and submucosa of each airway cross section (14, 27).

Experimental Protocol

After establishing a stable baseline Pb, DAC was performed in a sublobar segment ~15 min after pretreatment with either aerosolized saline, Mx, or NE. Baseline Pb was recorded after aerosol delivery, and colloidal carbon (1 ml/kg) was injected into the femoral vein 1–2 min after DAC was completed. Dogs were exsanguinated 15 min after DAC.

Results

A total of 134 airway cross sections from 9 dogs were examined. The average diameters of bronchi and bronchioles were 3.5 ± 0.11 (n = 54) and 1.8 ± 0.09 mm (n = 80), respectively. Pretreatment with vehicle and NE was done in four dogs; five dogs were pretreated with Mx. Neither vehicle nor NE aerosol significantly altered either mean arterial pressure (MAP; 153 ± 10 vs. 150 ± 11 mmHg; P = 0.885) or HR (153 ± 20 vs. 168 ± 16 beats/min, n = 4; P = 0.686). Similarly, pretreatment with Mx did not significantly affect MAP (127 ± 10 vs. 127 ± 11 mmHg, P = 1.0) or HR (162 ± 13 vs. 132 ± 17 beats/min, n = 5; P = 0.309). All measurements made on unexposed control airways from both groups were similar and were combined (n = 9) for statistical analyses.

Hyperventilation-Induced Mucosal Damage

The percent of airway perimeter occupied by C + G, C – G, and damaged mucosa in control and challenged bronchi and bronchioles is summarized in Table 1. Although DAC did not significantly increase dry air-induced damage in vehicle-treated bronchi, mucosal injury was significantly greater (P < 0.05) in NE- and Mx-treated bronchi compared with control bronchi (Fig. 1). No significant mucosal injury was evident in any DAC bronchioles compared with controls.

Hyperventilation-Induced Goblet Cell Secretion

The number of ciliated cells per millimeter of basement membrane in unchallenged control, DAC vehicle-treated, DAC NE-treated, and DAC Mx-treated bronchi were similar (Fig. 2). The number of goblet cells per millimeter of basement membrane was also similar for bronchi in all four groups: unchallenged control (26 ± 5 cells/mm²), DAC vehicle-treated (23 ± 6 cells/mm²), DAC NE-treated (24 ± 4 cells/mm²), and DAC Mx-
treated (24 ± 7 cells/mm²). The bronchioles showed similar trends. DAC did not alter G/C (Fig. 2).

Dry Air-Induced Changes in Mast Cell Number

The number of mast cells per square millimeter of lamina propria in control, vehicle-treated, NE-treated, and Mx-treated bronchi and bronchioles were similar (Fig. 3). No differences were detected in the number of submucosal mast cells in either bronchi or bronchioles. No significant differences were detected in mast cell number per square millimeter of lamina propria located below C₁G, C₂G, and damaged mucosa.

Dry Air-Induced Bronchovascular Hyperpermeability

Regardless of mucosal condition, negligible quantities of colloidal carbon were observed within the walls of vessels in the lamina propria of control bronchi and bronchioles (Fig. 4). Although not statistically significant, the density of colloidal carbon tended to increase from 117 ± 35 µm²/mm² in control bronchi to 577 ± 248 µm²/mm² in DAC-exposed vehicle-pretreated bronchi. Colloidal carbon was increased to 2,291 ± 704 and 4,092 ± 1,101 µm²/mm² (P < 0.05) in DAC-exposed bronchi pretreated with either NE or Mx, respectively. No significant difference existed between the NE- and Mx-treated airways. DAC did not significantly increase bronchovascular permeability in bronchioles.

Dry air-induced vascular leakage in the lamina propria was also examined as a function of the condition of the overlying mucosa regardless of airways size (Fig. 5). The density of colloidal carbon located below normal ciliated mucosa in control airways was 192 ± 53 µm²/mm². Of the three pretreatment groups, only Mx had colloidal carbon located below C₁G mucosa significantly increased above control levels (765 ± 238 µm²/mm², P < 0.05). Colloidal carbon located below C₂G mucosa in DAC-exposed vehicle-treated (984 ± 241 µm²/mm²) and Mx-treated (1,973 ± 1,035 µm²/mm²) airways were significantly increased when compared with normal mucosa in control airways (P < 0.05). Finally, extravasation of carbon beneath damaged mu-

![Fig. 1. %Airway perimeter containing dry air-damaged mucosa in control (Con), vehicle-treated (Veh), norepinephrine-treated (NE), and methoxamine-treated (Mx) bronchi (A) and bronchioles (B). Values are means ± SE. *P < 0.05 compared with control.

![Fig. 2. Ciliated cells/mm² (A) and goblet/ciliated cell ratios (B) in Con, Veh-, NE-, and Mx-treated bronchi and bronchioles. Values are means ± SE.

![Fig. 3. No. of mast cells/mm² of airway tissue in lamina propria of Con, Veh-, NE-, and Mx-treated bronchi (A) and bronchioles (B). Values are means ± SE.

Table 1. Effect of dry-air challenge on mucosal condition in canine airways

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C + G Mucosa, %</th>
<th>C - G Mucosa, %</th>
<th>Damaged Mucosa, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bronchi</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>54 ± 15</td>
<td>44 ± 14</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Vehicle + DAC</td>
<td>65 ± 18</td>
<td>31 ± 15</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>NE + DAC</td>
<td>37 ± 14</td>
<td>16 ± 8</td>
<td>47 ± 17*</td>
</tr>
<tr>
<td>Mx + DAC</td>
<td>35 ± 16</td>
<td>29 ± 12</td>
<td>36 ± 16*</td>
</tr>
<tr>
<td><strong>Bronchioles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>39 ± 11</td>
<td>60 ± 11</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Vehicle + DAC</td>
<td>26 ± 11</td>
<td>67 ± 12</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>NE + DAC</td>
<td>41 ± 15</td>
<td>50 ± 12</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>Mx + DAC</td>
<td>36 ± 14</td>
<td>61 ± 16</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE, in %airway perimeter containing ciliated and goblet cells (C + G), ciliated cells with few goblet cells (C - G), or damaged mucosa. DAC, dry-air challenge; NE, norepinephrine; Mx, methoxamine. *P < 0.05 compared with control bronchi.
sca in vehicle-treated (1,358 ± 258 µm²/mm²), NE-treated (1,860 ± 351 µm²/mm²), and Mx-treated (4,001 ± 146 µm²/mm²) airways was significantly increased when compared with normal mucosa in control airways (P < 0.05). Despite obvious trends, no significant difference between treatments was detected (P > 0.413). We previously reported that pretreatment with aerosolized Mx and NE reduces HIB in canine peripheral airways by ~25 and 60%, respectively (25). In that study, we concluded that NE reduced HIB primarily via the stimulation of β₂- and not α₁-adrenergic receptors. However, this finding does not exclude the possibility that NE-stimulated α₁-activity plays a role in other related events. In contrast, Mx did attenuate HIB via the stimulation of α₁-adrenergic receptors. We speculated that the modest inhibition produced by Mx resulted from either increased mucus secretion (reducing evaporative water loss and mucosal injury during DAC) or constriction of the bronchovascular tree (abating either hyperventilation-induced microvascular hyperperfusion, engorgement, bronchovascular leakage, or edema formation). The α-component of NE is likely to produce similar changes.

In this morphometric analysis, unexposed control airways were composed of normal ciliated epithelium and goblet cells, with an average of ~2% of the airway mucosa appearing damaged (Fig. 1, Table 1). Unlike in previous studies that used 5-min rather than 2-min exposure times (13, 14, 26, 27), DAC in the present study did not cause G/C (an indicator of goblet cell degranulation and mucosal perturbation) to decrease (Fig. 2). In fact, this 2-min DAC produced surprisingly little damage to the airway mucosa (Fig. 1). We used a milder stimulus in these experiments, because data from asthmatic subjects suggested that Mx provided protection only from very mild episodes of HIB (8). However, in contrast with our earlier speculations (25), pretreatment with either Mx or NE enhanced DAC-induced mucosal injury: ~36% of the perimeter of Mx-treated bronchi was injured, whereas ~47% of the NE-treated perimeter was damaged after DAC (Fig. 1). The extent of this mucosal injury is similar to our previously published results with the more potent 5-min stimulus (13, 14, 26, 27). Thus, assuming that Mx and NE do constrict the bronchial circulation (6, 20), the resulting reduction in bronchial blood flow is accompanied by a marked increase in damage to the bronchial mucosa (Fig. 1). This observation is consistent with our previous findings that 1) pretreatment with a β₂-adrenergic agonist, which presumably dilates the bronchial vasculature, significantly reduces hyperventilation-induced mucosal injury (26) and 2) impairment of bronchial blood flow enhances hyperventilation-induced mucosal injury in canine airways (13). All of these studies indicate that the bronchial circulation plays an important role in protecting the bronchial mucosa from local injury during hyperventilation, possibly by preventing dry air from entering the peripheral lung.

Unlike exposure to a 5-min DAC (13, 26, 27), 2 min of hyperventilation did not significantly alter mast cell numbers in any airway examined in this study. Treatment with neither Mx nor NE affected dry air-induced mast cell degranulation in any region of the airway (Fig. 3). Mast cell numbers did tend to be lower in Mx-treated bronchi, but this may reflect degranulation in response to direct α₁-receptor stimulation (19). Previous studies suggest that mast cell degranulation either contributes to or is a consequence of mucosal injury and plays an important role in the development of HIB (13, 14, 26, 27). However, because our morphometric analysis focuses only on completely degranulated mast cells, partial mast cell degranulation could account for the observed effects without any reduction in mast cell number.

Several studies have reported that α₁-agonists inhibit HIB in asthmatic subjects (8, 18) and suggest that these drugs do so by decreasing hyperventilation-induced microvascular hyperperfusion, engorgement, bronchovascular leakage, or edema formation. NE in particular is believed to inhibit the development of HIB in asthmatic subjects by reducing bronchial blood flow...
and, in so doing, reducing the rate at which airways rewarmed (18). However, human (23) and canine (12) airways respond similarly to hyperventilation, and changes in airway temperature in the absence of airway drying do not initiate HIB in dogs (12). These observations suggest that abrupt thermal changes are not a prerequisite for the initiation of HIB. Hyperventilation with dry air does increase bronchial blood flow in animals (2, 28) and results in bronchovascular hyperpermeability (13, 14). If either Mx or NE attenuated HIB by reducing bronchial blood flow, then the bronchovascular leakage that accompanies HIB should also be reduced. Although the milder stimulus used in this study did not significantly increase vascular leakage in vehicle-treated airways, Mx and NE markedly enhanced the hyperventilation-induced extravasation of fluid compared with unchallenged control bronchi (Fig. 4).

Examination of mucosal-dependent vascular leakage reveals a striking and progressive increase in fluid extravasation occurring below normal ciliated mucosa replete with goblet cells (C + G), goblet cell-depleted mucosa (C − G), and damaged mucosa. This dose-response relationship between increasing level of perturbation (mucosal category) and the magnitude of bronchovascular leakage is most obvious for Mx- and vehicle-treated airways (Fig. 5). In contrast, the enhancing effect of NE seen when vascular leakage is quantified without regard to mucosal condition (Fig. 4) is no longer obvious when analyzed by mucosal category, suggesting that NE primarily affects vessels that reside in damaged regions. Although the differences between vehicle-, NE-, and Mx-treated airways are not statistically different, the positive trend seen in Fig. 5 clearly shows that neither Mx nor NE decreases bronchovascular permeability in DAC canine peripheral airways and may actually enhance it. This is most obvious in vessels located below normal (C + G) mucosa, where only Mx-treated airways exhibit a significant increase in microvascular leakage. The greater efficacy in all mucosal categories of Mx compared with NE may directly reflect the greater α1-specificity of this drug (25).

Our results are markedly different from those obtained in studies of guinea pigs, showing that α1-agonists inhibit microvascular leakage produced by histamine (29) and platelet-activating factor (5). This inconsistency may result from differences in either stimulus or species. In rats (21), inhaled Mx inhibits substance P-induced microvascular leakage, presumably by reducing bronchial blood flow. Although inhaled Mx does not affect bronchovascular leakage, it does enhance airway leakage when administered intravenously. Larrazá et al. (21) suggested that, under the latter condition, α1-agonist-induced systemic hypertension caused pulmonary congestion, which in turn resulted in airway leakage that was unrelated to any direct effect of Mx on bronchovascular permeability. However, increasing doses of Mx enhanced bronchovascular leakage in a dose-dependent fashion, despite similar drug-induced changes in MAP for each dose. The lowest dose did not effect leakage. On the basis of these observations, Mx appears to have a direct effect on bronchovascular permeability in rats; this effect is consistent with our findings. In our study, neither aerosolized Mx nor NE affected HR or MAP, suggesting that any effects attributed to α1-agonists are likely to be local. However, our goal was not to determine whether α1-agonists enhance bronchovascular permeability but to determine whether these agents inhibit HIB by decreasing bronchial blood flow and bronchovascular leakage. Figures 4 and 5 clearly demonstrate these agents do not.

We previously suggested that bronchovascular leakage occurs in concert with airway narrowing and protects the bronchial mucosa from excessive losses of heat and water (13, 14). Mucociliary clearance in asthmatic subjects was reduced during and increased after isocapnic hyperventilation with dry air (7). Hyperventilation with dry air does increase bronchial blood flow in animals (2, 3, 28) and, as seen in this study (Figs. 4 and 5) and others (10, 13, 14), results in bronchovascular hyperpermeability. Thus the movement of extravasated fluid into the airway lumen may account for the increased clearance reported in these studies. α1-Agonists can affect tracheobronchial blood flow (6, 20) and water transport across airway epithelia (4) and may attenuate HIB by stimulating a fluid-replacement mechanism. We have published similar results for furosemide (16), which may also attenuate HIB in part by enhancing dry air-induced bronchovascular leakage and increasing water delivery to airway tissues. This would theoretically reduce the strength of the osmotic stimulus produced during a DAC and diminish the magnitude of obstruction that subsequently develops.

In summary, although Mx and NE inhibit HIB, they do not do so by reducing dry air-induced mucosal perturbation and injury, mast cell degranulation, or bronchovascular hyperpermeability. The observation that α1-agonists increase dry air-induced mucosal injury and bronchovascular leakage supports the notion that water replacement through the bronchial circulation opposes the desiccating effect of hyperventilation with dry air. The proximal distribution of both airway mucosal injury and bronchovascular leakage suggests that bronchi are exposed to a greater desiccating burden than bronchioles. Furthermore, it seems likely that the augmentation of bronchovascular leakage observed in α1-agonist-treated bronchi arises as a consequence of mucosal injury. Whether or not the tendency of these α1-agonists to enhance dry air-induced microvascular leakage plays a significant physiological role in the inhibition of HIB remains in question. However, even modest changes in microvascular permeability may adequately compensate for water loss that normally occurs during hyperventilation. If this is correct, then future studies examining the effects of α1-agonists and other vasoactive drugs on airway fluid balance and water flux may provide insights into the mechanisms underlying the development and expression of hyperpnea-induced asthma. However, it is important to note that these drugs do enhance hyperventilation-induced
airway injury and as such appear to be unlikely candidates for long-term clinical application.

We thank Dr. Walter Ehrlich for providing critical review of an early draft of this manuscript, and we gratefully acknowledge the superb technical assistance of Judith Corum and Dick Rabold.

This work was supported in part by National Institutes of Health Grants HL-51930 and ES-03819.

Address for reprint requests: A. N. Freed, Div. of Physiology, 7006 Hygiene, The Johns Hopkins Univ., 615 North Wolfe St., Baltimore, MD 21205 (E-mail: afreed@jhsph.edu).

Received 13 March 1997; accepted in final form 11 August 1997.

REFERENCES


