Airway hyperresponsiveness induced by cationic proteins in vivo: site of action

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Homma, Toshiaki, Jason H. T. Bates, and Charles G. Irvin. Airway hyperresponsiveness induced by cationic proteins in vivo: site of action. Am J Physiol Lung Cell Mol Physiol 289:L413–L418, 2005. First published April 29, 2005; doi:10.1152/ajplung.00059.2005.—Major basic protein and other native cationic proteins increase airway hyperresponsiveness when administered to the luminal surface of the airways in vitro. To determine whether the same applies in vivo, we assessed airway responsiveness in rats challenged with both aerosolized and intravenously infused methacholine. We partitioned total lung resistance into its airway and tissue components using the alveolar capsule technique. Neither poly-L-lysine nor major basic protein altered baseline mechanics or its dependence on positive end-expiratory pressures ranging from 1 to 13 cmH₂O. When methacholine was administered to the lungs as an aerosol, both cationic proteins increased responsiveness as measured by airway resistance, tissue resistance, and tissue elastance. However, responsiveness of all three parameters was unchanged when the methacholine was infused. Together, these findings suggest that cationic proteins alter airway responsiveness in vivo by an effect that is apparently limited to the bronchial epithelium.

METHODS

Experimental preparation. We studied 41 male Sprague-Dawley rats (Harlan Sprague-Dawley; Taconic Labs, Germantown, NY) that were free of common species-specific pathogens. The animals were 20–30 wk old and weighed 328 ± 15 grams (mean ± SE). Animal experiments followed the guidelines of the Animal Welfare Act of 1984 and were approved by the Institutional Animal Care and Use Committee at the University of Vermont. The rats were anesthetized with 70 mg/kg pentobarbital sodium injected intraperitoneally, with injections containing one-third of the initial dose given on a scheduled basis throughout each experiment to maintain anesthesia. A tracheotomy was performed, and a snugly fitted tracheal cannula [PE-240: 1.67 mm inner diameter (ID), 2.42 mm outer diameter (OD), and 25 mm length] was inserted into the trachea. The rats were placed on a warming pad and mechanically ventilated with a volume ventilator (model 683; Harvard Apparatus, South Natick, MA) that delivered a tidal volume of 10 ml/kg at 60 breaths/min with a positive end-expiratory pressure (PEEP) of 5 cmH₂O. A femoral venous line was placed for fluid and drug administration. Hydration was maintained with intravenous saline infused at 2.0 ml/h.

A heated pneumotachograph (model 8410; Hans Rudolph, Kansas City, MO) was attached to the proximal end of the tracheal cannula to measure air flow (V), which was determined from the pressure drop across the pneumotachograph measured with a differential pressure transducer (MP-45, ±2 cmH₂O; Validyne Engineering, Northridge, CA). Volume was obtained by digital integration of V. Tracheal pressure (Ptr) was measured through a lateral tap in the tracheal cannula using a second Validyne pressure transducer. The resistance of the tracheal cannula external to the trachea (Rest) was 0.04 cmH₂O·ml⁻¹·s⁻¹ and was constant for V up to 95 ml/s. Rest was subtracted from measurements of total resistance (see below) to yield the resistance of the lung (Rₜ) alone. The dead space of the tracheal cannula together with the pneumotachograph was 0.58 ml.

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$R_L$, measured during conventional mechanical ventilation has been shown in a variety of species to include a significant contribution from the lung tissues, and this contribution has been shown to have a marked inverse dependence on ventilation frequency (31, 33, 34). Our goal in the present study was to understand how cationic proteins affect the resistance of the airways (Raw) specifically. We therefore needed a way of measuring Raw separately from $R_L$, and of determining how the responsiveness of Raw depends on lung volume. This was provided by the alveolar capsule technique (12, 18, 27, 34), which was used to measure regional alveolar pressure ($P_A$) in two locations simultaneously, one on the left upper lobe and the other on the right cardiac lobe. Capsules were installed by inflating the lungs to 20 cmH2O and then gluing the capsule flange to the pleural surface using cyanoacrylate glue (Super Glue; Loctite, Cleveland, OH). The region of pleura isolated by each capsule was then punctured to a depth of 3.0 mm with an electrocautery needle so that $P_A$ could be measured by a pressure transducer identical to that used to measure airway pressure. The transducer responses were confirmed to be symmetrical and linear. The catheters (15 cm, ID 1.67 mm, OD 2.42 mm) that connected the capsules to the transducers were semipliable to prevent distortion of the pleural surface by the capsules during breathing. $P_A$ measurements were considered to be valid if 1) the magnitude of the swing in $P_A$ visually matched that of Prr during slow tidal ventilation, 2) pulmonary elastance ($E_L$) estimated from Prr was within 10% of that estimated from $P_A$, and 3) the two tissue resistance (Rti) determinations from each alveolar capsules were within 15% of each other. Alveolar capsule patency was ascertained according to these criteria every 5 min throughout the experiment. Measurements of $P_A$ that failed the above criteria (~15% in those animals treated with cationic proteins) were discarded.

Methacholine (Sigma Chemical, St. Louis, MO) was stored desiccated at $-20^\circ$C. A stock solution of 50 mg/ml in saline was prepared fresh on the day of study and serial dilutions were made with bacteriostatic buffered 0.9% saline (PBS). Control injections were performed using the same saline.

**Experimental protocol.** After a stable ventilation pattern was established and an absence of leaks in the alveolar capsules was confirmed, two lung inflations to 20 cmH2O were administered to standardize lung volume history. After a further 2 min of regular ventilation, baseline recordings of Prr, V′, and the two PA were made at 20°C. A stock solution of 50 mg/ml in saline was prepared and serial dilutions were made with normal saline (PBS). Control injections were performed using the same saline.

The dose-response curves to inhaled methacholine for $R_L$, Rti, Raw, and $E_L$, following intratracheal instillation of saline, PLL, or MBP. These measurements were made before methacholine challenge and show no effect of any of the interventions. The dose-response curves to inhaled methacholine for $R_L$, Rti, Raw, and $E_L$, following intratracheal instillation of PLL and MBP are shown in Figs. 1 and 2, respectively. Both cationic proteins shifted the dose-response curves upward and to the left. These displacements were statistically significant for all four parameters (ANOVA, P < 0.05).

Figure 3 shows the dose-response curves obtained when methacholine was delivered intravenously. Compared with the aerosol dose-response curves (Figs. 1 and 2), the intravenous dose-response curves are steeper and reach greater maximal levels. However, in marked contrast to the results with inhaled methacholine, treatment with PLL had no significant effect [ANOVA, not significant (NS)] on the dose-response curves to intravenous methacholine.

Baseline mechanics varied substantially with PEEP in rats treated with an intratracheal instillation of saline (Fig. 4). As PEEP varied from 1 to 13 cmH2O, $E_L$ and Rti increased by 750 and 500%, respectively, while Raw fell by <10%. PLL treatment did not significantly effect the relationship between PEEP and the various lung mechanics parameters (ANOVA, NS).
DISCUSSION

The present study confirms previous reports from our laboratory (6, 7, 35) that intratracheal treatment with cationic proteins results in hyperresponsiveness to aerosolized methacholine (Figs. 1 and 2). Furthermore, we found that this hyperresponsiveness applies not only to Raw but also to Rti and Eti as measured by the alveolar capsule. These observations would seem to implicate an effect of cationic proteins both centrally and peripherally in the lung. However, before making such a conclusion, we need to remember that using the alveolar capsule technique to partition lung mechanics into its central and peripheral components is based on the assumption that the lung behaves in a homogeneous fashion (22, 23, 28–30). In fact, bronchoconstriction is an inherently heterogeneous process (2, 20, 24, 25), particularly following aerosol challenge (28). Indeed, in the present study we observed an increased failure rate in achieving uniformity of pressure swings between the capsules in animals pretreated with cationic proteins compared with normal controls. Thus, although a response in Rti can lead one to think that the lung tissues are somehow constricting (30), such a response is more likely to arise from heterogeneous airway narrowing (2, 21, 24). Increases in Raw, on the other hand, are difficult to attribute to anything other than narrowing of conducting airways, so the results of the present study clearly show that intratracheal PLL elevates the responsiveness of the central airways to inhaled methacholine. The corresponding increases in Rti therefore may largely reflect heterogeneity of airway narrowing.

The most interesting result of our study is the finding that although rats treated with intratracheal PLL were hyperresponsive to inhaled methacholine, responsiveness was entirely un-

Table 1. Baseline lung mechanics data before aerosolized challenge with methacholine (aerosol challenge study) or before intravenous challenge with methacholine (intravenous challenge study)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RL, cmH2O · ml⁻¹ · s⁻¹</th>
<th>Rti, cmH2O · ml⁻¹ · s⁻¹</th>
<th>Raw, cmH2O · ml⁻¹ · s⁻¹</th>
<th>EL, cmH2O/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>0.13 (0.01)</td>
<td>0.017 (0.001)</td>
<td>0.099 (0.008)</td>
<td>1.03 (0.16)</td>
</tr>
<tr>
<td>PLL</td>
<td>0.14 (0.01)</td>
<td>0.019 (0.004)</td>
<td>0.116 (0.018)</td>
<td>1.22 (0.13)</td>
</tr>
<tr>
<td>MBP</td>
<td>0.11 (0.01)</td>
<td>0.016 (0.002)</td>
<td>0.090 (0.007)</td>
<td>1.16 (0.21)</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Aerosolize challenge study</th>
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<tr>
<td>SAL</td>
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<td>PLL</td>
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<th>Intravenous challenge study</th>
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<td>SAL</td>
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<td>PLL</td>
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Values are means (SE). SAL, saline; PLL, poly-L-lysine; MBP, major basic protein; RL, pulmonary resistance; Rti, tissue resistance; Raw, airway resistance; EL, pulmonary elastance.

Fig. 1. Effect of saline (SAL) (○, dashed lines) or poly-L-lysine (PLL) (○, solid lines) on responsiveness to aerosolized methacholine. NB: the log scale. The methacholine dose-response curves for all measures of pulmonary mechanics were shifted leftward and upward after treatment with PLL when compared with treatment after SAL. Rl, lung resistance; El, pulmonary elastance; Raw, airway resistance; Rti, tissue resistance. Data points are means ± SE. ANOVA: *P < 0.005, **P < 0.01, ***P < 0.05.

Fig. 2. Effect of SAL (○, dashed lines) or major basic protein (MBP) (○, solid lines) on methacholine responsiveness. The methacholine dose-response curves for pulmonary mechanics were shifted upward after treatment with MBP compared with treatment after SAL. Data are means ± SE. ANOVA: *P < 0.02, **P < 0.05.
changed when the methacholine was delivered intravenously (Fig. 3). An agonist delivered intravenously should result in a bronchoconstrictive response that is independent of the epithelium (32). Our results thus provide in vivo confirmation of our previous in vitro finding (6, 7) that when PLL is administered to the airway lumen, the resulting hyperresponsiveness to methacholine occurs only when the challenging agonist has to traverse the bronchial epithelium to reach the underlying smooth muscle. This supports the notion that intratracheal cationic proteins induce hyperresponsiveness by affecting the function of the epithelium in some as yet undefined way (7). Our results also indicate that the cationic proteins did not directly affect the smooth muscle itself, or indeed any of the elements comprising the pathway leading from the vasculature to the smooth muscle. In addition, the independence of lung mechanics on PEEP that we observed (Fig. 4) shows that PLL did not affect coupling of the airways to the parenchyma in which they are embedded. Uncoupling can occur, for example, as a result of peribronchial edema or destruction of alveolar walls (9, 26) and leads to a change in airway caliber through the release of the outward tethering forces exerted by the parenchymal attachments on the airway wall. Interestingly, we have previously shown that cationic proteins cause increased protein extravasation into the lung (5), but this was apparently not sufficient to cause airway-parenchymal uncoupling. Together, our results indicate that the intratracheal PLL had an effect that was limited solely to the bronchial epithelium.

The way in which cationic proteins affect epithelial function may be related to their high charge, as simple repetitive polycations such as poly-l-arginine or PLL are able to mimic the effects of MBP in inducing bronchial hyperresponsiveness (35). Also, hyperresponsiveness is attenuated when cationic proteins are neutralized by copresentation with anions such as heparin (7), or when they are rendered chemically neutral (acetylated PLL) (35). Others have shown similar charge-related effects. For example, Barker et al. (1) showed in primates that MBP increases airway responsiveness, but not when administered in conjunction with polylutamic acid. Fryer and Jacoby (15) have reported in guinea pigs that the MBP-induced change in airway neural function can be blocked by change neutralization. We have also previously shown that the electrical conductivity of a cultured epithelial layer is increased when treated with PLL and poly-l-arginine (36), suggesting that cationic proteins are able to disrupt the physical integrity of the epithelial membrane. This would, in turn, be expected to induce bronchial hyperresponsiveness by making the underlying airway smooth muscle more accessible to agonists present in the airway lumen (36).

The disruption of epithelial integrity in vivo is also supported by our previous findings of increased protein extravasation, as measured by Evans blue dye leakage around the airways, 15 min after PLL administration (8). Also, the damage to the epithelium appears to be transient as we have previously shown that methacholine responsiveness returns to normal 48 h after administration of cationic proteins (35). Before epithelial repair, however, it is easier for methacholine to move across the damaged epithelium, but our data suggest that the movement of plasma protein molecules in the opposite direction is not significantly increased because this would impair surfactant function. The result would then be an unstable lung that is more responsive to bronchial challenge (38). Furthermore, this
responsiveness would manifest regardless of whether the challenging agonist was delivered intravenously or into the airways as an aerosol, such as is the case for lungs treated with antigen (19, 34). Our results show that this was not the case for intratracheal PLL treatment, suggesting that cationic proteins permeabilize the epithelium sufficiently to increase the flux of methacholine but do not allow reverse passage of the much larger plasma protein molecules. In any case, reduced surfactant function has a significant effect in the lung periphery (38) but would not be expected to have a significant effect on the patency of the conducting airways as assessed by alveolar capsule. Together, this evidence supports the notion that PLL alters airway responsiveness by a mechanism altogether different from that of antigen.

The importance of cationic proteins for asthma stems from the fact that they are a principal product of eosinophils. These cells have been shown to accumulate in large numbers in the bronchial mucosa in hyperresponsive and asthmatic individuals (10, 16, 37, 39) and have been implicated in asthma pathogenesis by numerous studies. For example, eosinophil counts and eosinophilic cationic protein (ECP) and MBP levels in bronchoalveolar lavage fluid correlate with the severity of asthma (39). Also, MBP has been found on damaged epithelial surfaces and in mucus plugs in patients who died from status asthmaticus (16), showing that severe asthma is associated with a substantial presence of eosinophils and MBP within the airway lumen. Indeed, Clark et al. (4) recently showed that the majority of eosinophil degranulation predominately occurs in the lumen of the airways. Furthermore, we have previously shown in an airway tube system that cationic proteins also disrupt epithelial function when present to the basolateral aspect of the tissue but do not affect airway responsiveness when presented to the outside of the airway (7). MBP has also been shown to abolish ciliary activity of respiratory epithelium (13, 14, 17) and to have toxic effects on guinea pig (14, 17), rabbit (36), and human (14) respiratory epithelium in vitro. In addition, evidence of damage to the airway epithelium is a frequent finding in patients with bronchial asthma and is thought to be important in the development of airway hyperresponsiveness (3). This is evidenced by the fact that mechanical removal of the airway epithelium results in increased responsiveness to a variety of agonists (3, 7, 11), and increased numbers of epithelial cells in the bronchoalveolar lavage fluid has been shown to correlate with the degree of airway responsiveness (3, 39). Thus there is considerable evidence to suggest that epithelial damage by cationic proteins contributes to the airway hyperresponsiveness of asthma and that it is specifically the intraluminal presentation of cationic proteins that is key to their ability to enhance responsiveness. Also, given that cationic proteins appear to have a somewhat selective effect on the epithelium, the measurement of MBP, ECP, etc. in the lavage or sputum of patients is of particular relevance.

In summary, we found that intratracheal instillation of cationic proteins renders the lungs of rats hyperresponsive to methacholine when the antigen was inhaled, but not when it was delivered intravenously. Also, the PEEP dependence of baseline lung mechanics in the PLL-treated animals was identical to controls. We conclude, therefore, that when cationic proteins such as PLL and MBP are applied to the epithelial surface of the airways, they alter airway responsiveness in vivo solely by altering the function of the epithelium. A possible mechanism for this effect is a reduction in the barrier function of the epithelium, perhaps by reducing its ability to act as a barrier. This would be expected to make the underlying smooth muscle more accessible to agonists in the airway lumen. Our results offer an explanation for how cationic proteins might lead to bronchial hyperresponsiveness, when they are generated by inflammatory processes within the lung and able to reach the airway lumen.

REFERENCES