Methacholine responsiveness of proximal and distal airways of monkeys and rats using videomicrometry

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RAT AND MONKEY ARE BOTH SPECIES that are used as models of human airway hyperresponsiveness. However, the wall structures of rat and monkey airways are very different from each other, with that of the monkey more closely resembling that of humans. We hypothesized that differences in wall structure would explain differences in airway responsiveness. Using videomicrometry, we measured airway luminal area in lung slices to compare proximal and distal airway responsiveness to methacholine in the rat and monkey. The airway type was then histologically identified. Proximal airways of the young rat and monkey were equally responsive to methacholine. In contrast, respiratory bronchioles of monkeys were less responsive than were their proximal bronchi, whereas the distal bronchioles of rats were more responsive than their proximal bronchioles. Both proximal and distal airways of younger monkeys were more responsive than those of older monkeys. Airway heterogeneity in young monkeys was greatest with regard to degree of airway closure of respiratory bronchioles. We conclude that responsiveness to methacholine varies with airway wall structure and location.

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ticularly interested in evaluating whether the respira-
tory bronchioles of the monkey would show significant
narrowing and how they would compare with the distal
bronchioles of the rat. We hypothesized that the known
differences in wall structure of these airways would
help explain differences in proximal and distal airway
responsiveness in the two species.

MATERIALS AND METHODS

Animals. Sprague-Dawley female rats (n = 7) were ob-
tained from Zivic-Miller (Zelienople, PA) and utilized in these
experiments at 45–55 days of age. Rats were received at 4–5
wk of age and housed in filtered-air rooms for at least 1 wk
before necropsy. The rhesus monkeys (Macaca mulatta; n = 28)
used in this study were born at the California Regional
Primate Center colony and represented animals from three
different ongoing projects at California Regional Primate
Center. The ages of young monkeys were 30 days (n = 3), 70
days (n = 8), 90 days (n = 3), and 6 mo (n = 8). Six 3-yr-old
monkeys were also used. With the exception of four female
70-day-old monkeys, all monkeys were males. The young
monkeys were housed from birth indoors in filtered-air
rooms, with the exception of two 6-mo-old monkeys that were
housed out of doors. Seventeen monkeys’ skin tested nega-
tively for dust mite. Three of the 6-mo-old and eight of the
70-day-old monkeys were not skin tested. All 3-yr-old mon-
keys were field controls, housed in outdoor field corrals. Care
and housing of animals complied with the provisions of the
Institute of Laboratory Animal Resources and conformed to
practices established by the American Association for Accred-
itation of Laboratory Animal Care.

Tissue preparation. Rats were anesthetized with pentobarb-
ital sodium (1 mg/kg ip). A ventral incision in the neck was
made to cannulate the trachea, the chest was opened, and
heparin (100 U) was injected into the right ventricle. The
pulmonary artery was cannulated via the right ventricle, the
left ventricle was incised, and 50 ml of warm (37°C) Krebs
buffer with 2% bovine serum albumin were perfused through
the lungs. The lungs and heart were removed en bloc from
the thoracic cavity, and warm (37°C) Krebs buffer without
methacholine. Drug concentrations started at
10\textsuperscript{−8} M (rat) or 10\textsuperscript{−9} M (monkey), with each subsequent
concentration increasing by at least one-half log increments
to a maximum 10\textsuperscript{−4} M. For each concentration, the old
airways were continually aerated with 95% O\textsubscript{2} and 5%
CO\textsubscript{2} (carbogen), at pH 7.35 ± 0.03. Solutions were aerated
with carbogen for at least 5 min, and pH was measured before
application to slices. Preliminary studies showed that
pH was constant after 5 min of aeration with carbogen. After
a minimum of 40 min, with Krebs buffer changes every 5
min, airways with a luminal area changing <10% between
washes and pulsating <10% by visual inspection were chal-
lenged with methacholine. Drug concentrations started at
10\textsuperscript{−8} M (rat) or 10\textsuperscript{−9} M (monkey), with each subsequent
concentration increasing by at least one-half log increments
to a maximum 10\textsuperscript{−4} M. For each concentration, the old
solution was removed and the new one added. An image was
captured 5 min after each buffer change and after 5 min of
incubation in each concentration of drug. Preliminary studies
with rats and monkey slices found the responses to drug to be
stable between 2 and 8 min of incubation time. Preliminary
experiments showed that, when this procedure was per-
formed with Krebs buffer without methacholine, the luminal
area was stable in both rats and monkeys. Distal and prox-
imal airways were paired in the image-capture protocol, to
avoid any time delay effects in comparing proximal vs. distal
responsiveness.

Histological evaluation of the airways. After measurement
of airway response, all lung slices were fixed in 1% parafo-
maldehyde for a minimum of 24 h before being embedded in
paraffin (Paraplast-20, Oxford Labware, St. Louis, MO). Se-
rial sections (5 μm thick) were cut by using a microtome (Carl
Zeiss, Thornwood, NY) and stained with hematoxylin and
eosin. The airway within each lung slice was examined by
light microscopy. The alveoli appeared to be properly inflated
and of uniform size. The airway epithelium showed no evi-
dence of damage from removal of the agarose plug. Airways
were identified by structural features. A bronchus was iden-
tified by the presence of cartilage in the wall, a bronchiole
by the lack of cartilage or alveolar outpocketings within the
wall, and a respiratory bronchiole by alveolar outpocketings
along the wall.

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Reagents and solutions. SeaPlaque agarose was obtained from FMC Bioproducts (Rockland, ME). Waymouth mouth MB 752/1 medium was purchased from GIBCO BRL, Life Sciences (Grand Island, NY). Sodium bicarbonate (2.24 mg/l) was added to the Waymouth solution, and pH was adjusted to 7.4 at 4°C. The Krebs buffer (119 mM NaCl, 4.7 mM KCl, 3.2 mM CaCl2, 21 mM NaHCO3, 1.17 mM MgSO4·7H2O, 1.18 mM KH2PO4, and 0.1% D-glucose; Mallinkrodt, Paris, Kentucky, and Sigma Chemical, St. Louis, MO) was made fresh weekly and heat sterilized. NaHCO3 and CaCl2 were added on the day of the experiment; the buffer was aerated with carbogen and adjusted to a pH of 7.4 at 37°C. Final concentrations of methacholine (acetyl-β-methylcholine chloride; Sigma Chemical) were prepared fresh on the day of each experiment in Krebs buffer from 10⁻² M stock. Nexaband was purchased from Veterinary Products Laboratories, and bovine serum albumin was purchased from Sigma Chemical.

Data analysis and statistical evaluation. The epithelial luminal border of the airway was traced, and the luminal area was calculated by using NIH Image software. The data were expressed as a percentage of the lumen measured in the last buffer wash. Concentration-response curves were compared by using a repeated-measures analysis of variance. When appropriate, post hoc analyses consisted of a series of Scheffe contrast tests among the treatment groups (SAS/Stat, SAS Institute).

Heterogeneity of airway responsiveness to methacholine, as indicated by maximal response and the concentration of methacholine that decreased the luminal area to the midway point between the areas at control and maximal response (EC50), was evaluated in monkeys 6 mo of age and younger. The younger monkey database was chosen because it had a sufficient number of animals with multiple slices to evaluate intra-animal variance. For evaluation of intra-animal heterogeneity, airways were included if at least two airways from the same region (proximal or distal) in the same animal were available. The maximal response, defined as the airway luminal area at maximal closure, was expressed as a percentage of original luminal area. EC50 was calculated by linear interpolation between log-transformed methacholine concentrations. The individual animal variances of these values in the proximal and distal airways were compared by using a t-test.

Data were log transformed if variances differed by more than threefold. Significance was defined as a P value <0.05.

RESULTS

Videomicroscopy technique. Water-immersion lenses provided excellent visualization of the epithelial luminal border as airway smooth muscle constricted, as shown in Fig. 1. In addition, some of the airways in the monkeys were noted to pulse by spontaneously opening and partially closing in an ~3-s cycle, with ~0.5 s for constriction and 2.5 s for relaxation. This pulsation was not affected by addition of indomethacin (10⁻³ M). If the pulsation was significant, the slice was not used for data acquisition. If the pulsation was minor (~10% by visual inspection), the image was captured in the most open configuration.

In an effort to reduce variability in the data, the effects of obliquely cut vs. cross-sectionally cut airways on concentration-response curves were evaluated. As shown in Fig. 2 for distal airways, the obliquely cut airways were less reactive to methacholine and closed less completely than cross-sectionally cut airways. Thus only cross-sectionally cut airways were used for data analyses.

Histological evaluation of the airways. By histological evaluation of the monkey lung slices, it was determined that 95% of the proximal airways were bronchi, with the presence of cartilage in the wall and intact epithelium lining the lumen. Ninety-five percent of distal airways in monkey lung slices were found to have alveolar outpocketings in the walls and were defined as respiratory bronchioles. The remaining airways were labeled as bronchioles and were typically within one to two generations of a respiratory bronchiole. In contrast, rat lung slices contained only bronchioles, with large bronchioles identified in proximal slices and small bronchioles identified in distal slices. In all instances for both species, the epithelial lining of all airways was found to be intact.

Comparison of 7-wk-old rat and 6-mo-old monkey proximal and distal airways. Histological evaluation of the proximal airways (Fig. 3, Table 1) from the lung
slices showed the expected morphology for the rat and monkey airways. Specifically, the monkey proximal airway had pseudostratified columnar epithelium, cartilage, goblet cells, and submucosal glands, whereas the rat had simple columnar epithelium and no cartilage, goblet cells, or submucosal glands. In the distal airways, the monkey had alveolar outpocketings indicative of respiratory bronchioles, whereas the rat had bronchioles without alveolar outpocketings. In airway slices used in the comparison, the rat proximal airways were approximately the same diameter as those from the monkey, whereas the rat distal airways were bigger than those from monkey. All airways, independent of size and type, demonstrated the presence of interrupted, circumferential bands of smooth muscle segments. Proximal airways for both monkeys and rats possessed prominent bands of smooth muscle, whereas distal airways contained fewer. Physiological evaluation (Fig. 4) showed that the rat and monkey proximal airways were equally responsive to methacholine. However, rat distal airways were more responsive to methacholine than monkey distal airways. In the rat, the distal airways were more responsive to methacholine than the proximal airways, whereas, in the monkey, the reverse was observed: distal airways were less responsive to methacholine than proximal airways.

**Comparison of monkey proximal and distal airways at different ages.** Airway responsiveness to methacholine was evaluated in monkeys at various ages. Because airway responsiveness in monkeys 6 mo of age and younger did not differ, the data were combined and compared with data from airways of 3-yr-old monkeys. The proximal and distal airways of young monkeys were more responsive than those of older monkeys (Fig. 5).

**Intra-animal heterogeneity in airways from young rhesus monkeys.** Intra-animal heterogeneity of airway responsiveness to methacholine is shown in Fig. 6. The mean of the individual animal variances for the EC50 in the proximal airways (0.24 ± 0.13; n = 13) did not differ statistically from that in the distal airways (0.28 ± 0.12; n = 11; P = 0.42). However, for the maximal response, the mean of the individual animal variances was 1.27 ± 0.42 (n = 13) in the proximal airways and 1.92 ± 0.48 (n = 11) in the distal airways (P < 0.001). The mean of the individual animal variances for the maximal response in the proximal airways was not significantly different from that in the distal airways (P = 0.12). Therefore, the heterogeneity in airway responsiveness to methacholine in young rhesus monkeys was not extensive.
variances was much less in the proximal airways (31 ± 12) than in the distal airways (401 ± 259; \( P < 0.02 \)).

**DISCUSSION**

By using videomicrometry in conjunction with lung airway slices, a method that allows measurement of airway contractility by individual generations with intact peribronchial constituents, we found variability by airway generation, age, and species. This study is the first to compare the responsiveness of proximal and distal airways of monkeys and rats. In fact, to the best of our knowledge, this represents the first time the responsiveness of the respiratory bronchiole, as defined by its anatomic structure, has been measured in any species. We demonstrated that the rat and monkey proximal airways used in this study were remarkably similar in their response to methacholine. In contrast,

![Table 1. Comparison of rat and monkey airway slice morphology and histology](image)

Body weight and lumen diameter values are means ± SE. *Significantly smaller than rat distal bronchioles, \( P < 0.01 \).
Fig. 6. Intra-animal heterogeneity of airway responsiveness to methacholine in young monkeys. If at least 2 airways from the same region (proximal or distal) in the same animal were available, the concentration in log mmolar units of methacholine that produced 50% of the maximal response (EC50) and the maximal response were determined. ○, data from one airway slice. A and C: frequency distribution of EC50 in proximal (n = 13 animals) and distal airways (n = 11 animals). B and D: frequency distribution of the maximum response in the proximal and distal airways. One hundred represents the original luminal area, and zero represents an airway that is completely closed. Whereas the variances of the EC50 within each animal did not differ between proximal and distal airways, the variances of the maximal response were less in the proximal than in the distal airways (P = 0.02).

rat distal bronchioles were more responsive than proximal airways to methacholine, whereas, in the monkey, respiratory bronchioles (i.e., distal airways) were less responsive than bronchi (i.e., proximal airways). In addition, both proximal and distal airways from young monkeys were more responsive to methacholine than those from 3-yr-old monkeys.

An extensive database exists to identify differences between the rat and primate airway wall structure (23, 26). Proximal airways in the rat differ from those of the monkey by the presence of a simple cuboidal or columnar epithelium, rather than pseudostratified epithelium. In addition, proximal airways of the rat lack goblet cells, submucosal glands, and cartilage. The presence of cartilage in the proximal airways of the monkey might be expected to limit the narrowing of the airway lumen. Jiang and Stephens (10) showed that bronchial smooth muscle without cartilage attached demonstrated greater maximum shortening capacity and greater maximum velocity of isotonic shortening than bronchial smooth muscle with cartilage intact. In the present studies, virtually all of the proximal airways studied from monkeys contained cartilage. However, the presence of cartilage did not appear to inhibit proximal airway responsiveness.

Distal airways in the rat differ from those in the monkey to an even greater degree than do the proximal airways (Table 1). In the rat, up to 16 airway generations are present before reaching the terminal bronchi-ole, immediately giving rise to the alveolar duct (26). In the monkey, there are up to 16 generations of airways (personal communication, Michelle Fanucchi, University of California, Davis), which subsequently give rise to three to six generations of respiratory bronchioles before reaching the level of the alveolar duct (23). In this study, virtually all distal airways in monkeys were identified anatomically as respiratory bronchioles. Despite the interruption of the wall with outpocketings of alveoli, the monkey respiratory bronchioles were responsive to methacholine. However, as shown statistically in the younger monkeys, the respiratory bronchioles were the least responsive and most variable airway studied. There was no obvious histological explanation for the hyperresponsiveness of rat distal airway compared with the proximal airway. Possible mechanisms include fewer infolds (internal tethers); thinner lamina propria and submucosa (21); thick epithelial basal lamina (24); different smooth muscle area, configuration, or cell biology; less diffusion distance (18); and increased epithelial permeability (18). Recently, Wohlsen et al. (25), using videomicrometry techniques, showed that smaller airways of Wistar rats were more responsive to allergen and ketanserin, a serotonin antagonist, than were larger airways. Similarly, Mitchell and Sparrow (18) demonstrated, in pig airways in vitro, that small-lumen (3 mm2) bronchial tubes were more sensitive to methacholine and closed more completely than did larger lumen bronchial tubes (18 mm2). On the other hand, Minshall et al. (17), using videomicrometry techniques similar to those used in the present study to examine human airways from lung tissue resected for cancer, did not see a difference in responsiveness between large and small airways. However, differences between proximal and distal airways may have been obscured, because the airways came from different lobes and parts of lobes and because the airways were inflamed as a result of cigarette smoke exposure. Thus it may be that, in general, airway responsiveness increases in the distal airways compared with the proximal airways but that, once the airways become respiratory bronchioles as they do in the human and monkey, airway responsiveness is less.

A number of studies in rats (12), rabbits (22), and humans (3, 7, 14) have suggested that airway respon-
siveness is greater in younger than in older animals. We previously showed that the isolated perfused lungs from 8-wk-old rats were two- to threefold more responsive to methacholine at the highest dose given than were those from 15-wk-old rats (12). This is the first study to compare the developmental aspects of airway reactivity of proximal and distal airways in the monkey. We showed that both proximal and distal airways were more responsive to methacholine in younger than in older primates.

The observation of pulsing airways in monkeys (rarely found in rats) was an unexpected and interesting phenomenon. This pulsation was not due to cyclooxygenase products, because indomethacin did not inhibit this unique feature. In vivo airway physiology in primates may be even more dynamic than had been previously appreciated.

It is generally thought that airways within a person or animal show heterogeneity in responsiveness. In fact, airway heterogeneity is one of the potential explanations for the increase in Rti with methacholine administration (13). The study of human airways by Minshall et al. (17) reported marked heterogeneity in human airways by using videomicrometry techniques. Although we also saw some heterogeneity in responses in the young monkeys, it was much less than they reported. Our intra-animal variances for EC_{50} in both proximal and distal airways, and for maximal effect in the proximal airways, were fivefold less than that reported by Minshall et al. However, we did see marked variability in maximal effect in the distal airways. The variance of these airways was 12-fold that in the proximal airways. Thus, although heterogeneity exists, we believe it is mostly confined to the degree of closure of respiratory bronchioles.

We believe that improvement in technique is the reason we showed less heterogeneity than has been reported by others. We have made a number of adjustments in our technique to reduce variability. We always studied generations within the axial airway of the lobe. We cut our slices with a vibratome to a consistent thickness (600–650 μm) that would allow for clearly defined airway lumen and also be thick enough for histology procedures. We glued the edges of the lung slice to a coverslip to optimize the intrinsic tethering forces of the surrounding parenchyma. This is important because in vivo studies have shown that tethering of the airways affects airway responsiveness to spasmogens (1, 2) and Mitchell et al. (19) showed in vitro in lung slices that bronchoconstriction will stretch adjacent lung parenchyma, imposing a load on airway smooth muscle. Although the concentration of agarose can affect the closure of airways (5), we used the same concentration (1.25%) in all our studies. Inflation volume with the agarose can also affect airway closure (5). We used a standard method to fill the lungs with agarose, which was done by the same person for every experiment. Histological examination showed the alveoli to be equally distended. A water-immersion lens, rather than an inverted microscope (5, 15), allowed for a more precise image of the airway. We also extended our prewashes until the luminal area of the airway was changing by no more than 10%, and we rejected airways that failed to stop pulsating. Finally, as shown in Fig. 2, eliminating obliquely cut slices provided much more consistent data, especially for distal airways.

In order for comparisons to be made within and between species, there must be consistency in the method for airway selection. This was accomplished by (1) studying airways from a lobe with a single primary axial airway, (2) localizing the distance along the lobe (proximal or distal), and (3) describing the airway's luminal size and structure as shown by histology. We were certain that the proximal airways were proximal to the distal ones. In addition, in the monkey, we knew that the proximal airways were histologically bronchi or bronchioles, whereas the distal airways were almost entirely respiratory bronchioles. In the rat, this histological verification was not possible, because the proximal and distal airways in the rat appear similar until the terminal bronchiole. Although airway luminal size does not correlate well with airway generation, in general, the distal airways were smaller than the proximal airways, as would be expected. With regard to the lobes, we picked the lobe from each species that would provide the straightest axial airway. In the monkey, the accessory lobe axial airway curves sharply at first and then straightens out and divides in a monopodial fashion until, in the final one-fourth of the lobe, it begins to divide dichotomously. In the rat, the infracardiac lobe airway has a less sharp curve proximally and remains monopodial throughout the lobe. A large axial airway throughout the lobe probably explains why the distal airways of the rats were larger than those of the monkey, despite coming from the same relative section of the lobe.

Other potential methodological concerns in the between-species comparisons are differences in drugs used at necropsy (heparin in the rat and ketamine in the monkey) and viability. With regard to the differences in drugs at necropsy, the vasculature was cleared of drug by buffer perfusing the rats and exsanguinating the monkeys. The slices were then washed eight times for a total of at least 40 min before the study. We do not believe that residual drug remained in the tissues with this procedure. Finally, we believe all airways were viable. The proximal airways of both species and the distal airways of the rat all closed by at least 40% to methacholine, suggesting that they were viable. Although some of the distal airways of monkey did not show as much response to methacholine, these slices were handled identically. Further evidence of viability of the tissue is that on histological evaluation the epithelium was intact, without blebbing, vacuolization, or sloughing.

Videomicrometry is a useful tool to study differences in airway physiology. Recently, Martin et al. (16) used videomicrometry elegantly to show that endothelin-1 affects small and large airways in the rat to the same extent, whereas a thromboxone agonist was 10-fold more potent in contracting small airways compared...
with large airways. Our present work demonstrates that this technique of studying the responsiveness of a distinct airway can be coupled with evaluating the wall structure of that same airway. This attribute of our videomicrometry method will also make it useful in studying toxicological, allergic, and infectious influences that differ by airway generation. Such site-specific differences among airways have been shown after exposure to ozone on airway epithelial cell injury (11), as well as glutathione levels (6). Furthermore, within a generation, there may be focal injury (20). Thus combining airway physiology with histology in the same slice will allow for studying such airway changes.

In conclusion, we have shown that videomicrometry is a powerful tool for evaluating responsiveness of individual intrapulmonary airways. This is especially true for the smaller distal airways, which are difficult to study by other techniques. Furthermore, this technique allows for selective study and comparison of proximal and distal airways. More specifically, in the present study, we have shown that the respiratory bronchioles of the monkey were less responsive, and the distal bronchioles of the rat were more responsive, than the proximal airways of either species. In the monkey, both proximal and distal airways were more responsive in younger than in 3-year-old animals. We have shown that heterogeneity in airways is less than previously thought except for maximum closure of respiratory bronchioles. Most importantly, we have shown that respiratory bronchioles can be studied as distinct airways, that they are responsive to methacholine, and that they differ in their responsiveness from that of the most distal airway of the rat. This adds to the evidence that the monkey is a better experimental model for human airway responsiveness. Use of videomicrometry methods in combination with histological evaluation will greatly facilitate our understanding of the constitutive function of the airways and allow for better investigation of effects of toxins and allergens on specific airway generations.

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