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Temporal variability in the responses of individual canine airways to methacholine

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Abstract

Previous work showed that individual airway size, before any spasmogen, varied widely in the same animals on different days. The effect of this variable baseline size on the airway response to a subsequent challenge is unknown. The present study examined how the variability in individual airway baseline size in dogs was related to that after methacholine challenge on 4 different days using high-resolution computed tomography scans. Dogs were anesthetized and ventilated, and on 4 separate days randomly varying between 1 and 8 wk apart, baseline scans were acquired, followed by a continuous intravenous infusion of methacholine at three rates in increasing order (17, 67, and 200 $\mu\text{g}/\text{min}$). As the measure of variability, we used the coefficient of variation (CV) of the four airway luminal measurements of each airway at baseline and at each dose of methacholine. For most airways, there was wide variability both between and within dogs in the response to a given dose of methacholine ($\text{CV} = 33\text{--}38\%$). Airways with any level of methacholine stimulation had greater variability than those at baseline. The airway variability was greatest at the lowest dose of methacholine administered but was elevated at all the doses. In conclusion, there was substantial day-to-day variability in baseline airway size. Most importantly, the same dose of methacholine to the same individual airway showed even greater variability than that at baseline. If we consider that increased heterogeneity may potentiate clinical symptoms, then airway response variability may play an important role in the manifestation of airway disease.

Keywords

airway smooth muscle; asthma; heterogeneity; vagal tone; airway responsiveness

Airways are never static. Airways regularly dilate and narrow passively during normal changes in lung volume with breathing. In addition, there are active changes in the size of the airways from smooth muscle contraction. This active narrowing may be minor or can become excessive and cause wheezing and difficulty breathing as occurs during asthma attacks. Although all airways have some degree of baseline tone (10), the level of this tone is

generally not known, nor is its effect on any further contraction of the airway smooth muscle.

In previous work, we showed that the size of individual airways at baseline in dogs, before the administration of any spasmogen, varied widely in the same animals on different days over weeks and months (14). The dogs were studied five to eight times over the course of a year. We concluded that there was substantial local variability in the airway caliber at baseline and speculated that it may contribute to the local pathogenesis of lung disease (14). However, there was no follow-up study to test this speculation.

With normal assessments of airway responsiveness, the sole factor determining the caliber of the contracted airway is the dose of spasmogen administered. Airway responses are given as percentages of that in the baseline state. However, given the day-to-day variability in the size of the airway at baseline, the resulting size of the exogenously contracted airway may be dependent on the baseline size. Whether airways that are more contracted at baseline will contract more during exogenous stimulation is completely unknown. Therefore, we undertook the current study to examine how the variability in individual airway size in dogs at baseline is related to that after methacholine (MCh) challenge on 4 different days using high-resolution computed tomography (HRCT) to measure airway size.

Methods

The study protocol was approved by the Johns Hopkins Animal Care and Use Committee. Five dogs weighing ~20 kg were anesthetized with thiopental (15 mg/kg induction dose followed by 10 mg·kg⁻¹·h⁻¹ iv maintenance dose). After induction of anesthesia, the dogs were paralyzed with 0.5 mg/kg of succinylcholine with occasional supplemental doses as required to ensure no respiratory motion during imaging. Following endotracheal intubation with an 8.0-mm internal diameter endotracheal tube, the dogs were placed supine, and their lungs were ventilated with room air with a volume-cycled ventilator (Harvard Apparatus, Millis, MA) at a tidal volume of 15 ml/kg and a rate of 18 breaths/min. A stable depth of anesthesia was maintained by monitoring heart rate changes and eyelash reflex.

Protocol

Each dog served as its own control. The dogs were anesthetized and ventilated as described above. On 4 separate days randomly varying between 1 and 8 wk apart, baseline HRCT scans were acquired (see *Imaging and analysis of airways*), and the dogs then received a continuous intravenous infusion of MCh at three rates in increasing order (17, 67, and 200 µg/min; Sigma Chemical, St. Louis, MO); the middle dose, 67 µg/min, was previously demonstrated to decrease the size of the airways to ~60% of baseline (13). After the completion of the final dose of MCh, intravenous atropine (0.2 mg/kg) was administered, a dose previously shown to completely block vagal tone in the dog (8). To standardize lung volume history, before the first scan series, the airway pressure was increased to 45 cmH₂O, held for 5 s, and then released, and the animals were ventilated normally. At each dose and after atropine, HRCT scans were acquired to measure airway areas.

Imaging and analysis of airways

HRCT scans were obtained with a Sensation-16 scanner (Siemens, Iselin, NJ) using a spiral mode to acquire ~300 CT images during an 8-s breath hold (apnea) at 137 kVp and 165 mA. The images were reconstructed as 1-mm slice thickness and a 512 × 512 matrix using a 175-mm field of view and a high spatial frequency (resolution) algorithm that enhanced edge detection, at a window level of -450 Hounsfield units (HU) and a window width of 1,350 HU. These settings have been shown to provide an accurate measurement of luminal size as

small as 0.5 mm in diameter (24, 44). For repeated airway measurements in a given dog within each experimental protocol, adjacent anatomic landmarks, such as airway or vascular branching points, were defined and used to measure the airway size at the same anatomic cross sections.

The HRCT images were analyzed using the airway analysis module of the volumetric image and display analysis image analysis software package (Department of Radiology, Division of Physiologic Imaging, University of Iowa, Iowa City, IA) as previously described and validated (1, 8). The HRCT images were transferred to a Unix-based Sun workstation. An initial isocontour was drawn within each airway lumen, and the software program then automatically located the perimeter of the airway lumen by sending out rays in a spoke-wheel fashion to a predesignated pixel intensity level that defines the luminal edge of the airway wall. Intra- and interobserver accuracy and variability of the software program using this HRCT technique in phantoms, consisting of rigid tubes to measure known areas, have been previously shown by our laboratory (24) and by others (1) to be highly resistant to operator bias.

Data analysis

To assess airway variability, the coefficient of variation (CV) of the four airway luminal measurements of each airway at baseline and at each dose of MCh was calculated (the standard deviation of the 4 airway measurements divided by the mean of the 4 airway measurements $\times 100$) and used in a multivariate regression analysis as the dependent variable (JMP release 5.1; SAS Institute). The independent variables included the dose of MCh as an ordinal variable with four levels of 0, 17, 67, and 200 $\mu\text{g}/\text{min}$ and the dog as an indicator independent variable. Data were analyzed by one-way ANOVA with correction for multiple comparisons where appropriate, and simple linear and multivariate regression analyses were performed where appropriate. Significance was considered if the P value was <0.05 .

Results

A total of 312 airways for 5 dogs was matched and measured with the number of airways measured per dog ranging from 44 to 73. Airway size ranged from 2.3 to 21 mm in diameter. For all dogs, the mean relative airway size, defined as the percentage of the maximum area of the airway after atropine, was $77.5 \pm 7\%$ (means $\pm$ SD), $58.9 \pm 13\%$, $44.4 \pm 15\%$, and $32.5 \pm 12\%$ at baseline and during the MCh infusion of 17, 67, and 200 $\mu\text{g}/\text{min}$, respectively ($P < 0.0001$ for all pairwise comparisons).

When we compared the individual airway diameter at baseline on a given day with its diameter after MCh on the same day, there was a strong correlation. For all the doses of MCh, there were significant positive correlations between the diameter at baseline and during MCh at 17 $\mu\text{g}/\text{min}$ ($r = 0.93$, $P < 0.00001$; Fig. 1A), 67 $\mu\text{g}/\text{min}$ ($r = 0.91$, $P < 0.00001$; Fig. 1B), and 200 $\mu\text{g}/\text{min}$ ($r = 0.91$, $P < 0.0001$; Fig. 1C), respectively. Even with these high correlations, there was a wide range of responsiveness, especially in the middle of the baseline size range, as demonstrated by the vertical spread of airway responsiveness at each dose (Fig. 1, A–C).

To examine the extent of variability over different days, we calculated the overall CV, again calculated as the standard deviation of the four airway measurements divided by the mean of the four airway measurements $\times 100$, for all the airways in all dogs. The CV for the individual airways at baseline had a range between 0% and 28% and a mean of $10.9 \pm 7\%$. The mean CV for each dog ranged from 3.9% to 19.8% (Table 1).

During contraction, the variability of the airways increased. Furthermore, there were considerable differences in the changes in the CVs by dose among the dogs (Fig. 2). We saw the greatest increase in variability at the lowest MCh dose administered. The overall CV during contraction doubled, increasing from $10.9 \pm 7\%$ at baseline to $20.5 \pm 7\%$ during the MCh infusion of $17 \mu\text{g}/\text{min}$ ($P < 0.001$; see below). The CV for the individual airways at $17 \mu\text{g}/\text{min}$ of MCh ranged from a low of 3.9% to a high of 38%. The dog-specific mean CV at $17 \mu\text{g}/\text{min}$ of MCh for all airways varied from 13.7% to 24.5% (Table 1).

At $67 \mu\text{g}/\text{min}$ of MCh, the CV for the individual airways ranged from a low of 0% to a high of 33% with a mean of $14.3 \pm 7\%$. The dog-specific mean CV varied from 9.3% to 21.8%. At the highest dose of $200 \mu\text{g}/\text{min}$ of MCh, the CV for the individual airways at $200 \mu\text{g}/\text{min}$ of MCh ranged from a low of 0% to a high of 37% with a mean of $13.5 \pm 6\%$. The dog-specific mean CV varied from 8.0% to 17.0%.

Finally, to investigate whether the variability differed across doses and across dogs, we performed multivariate regression analyses using the CV for each airway as the dependent variable, the dog as an indicator independent variable, and the MCh dose as an ordinal independent variable. Overall, we found that there were differences in the CV between dogs and between doses. Specifically, when controlled for dogs, there was a significant difference in the CV between the baseline and the MCh dose of $17 \mu\text{g}/\text{min}$ ($P < 0.001$) and between the MCh dose of 17 and $67 \mu\text{g}/\text{min}$ ($P < 0.001$) but not between the MCh dose of 67 and $200 \mu\text{g}/\text{min}$ ($P = 0.13$). The CV for the 67 and $200 \mu\text{g}/\text{min}$ dose of MCh also remained significantly higher than the CV at baseline ($P < 0.001$). With the MCh dose controlled, there were significant differences in the CV among the dogs; however, not all pairwise comparisons were significant (Table 2).

Discussion

In this study, we measured the size of individual airways on repeated occasions at baseline and in responses to the same doses of MCh. As far as we are aware, there are no comparable data in the literature that have examined this temporal variability in airway responsiveness. Although, as will be discussed in *Literature studies relevant to airway heterogeneity*, there have been studies that have examined repeated measurements of pulmonary function in humans, these do not provide any insights in the spatial variability. In addition, although the temporal variability in baseline airway size was previously studied (10), the response to exogenous challenge was not tested.

Before a discussion of our data and its interpretation, it is worth considering a more general discussion of airway responsiveness. When MCh is given, it is well known that airway smooth muscle contracts and the airways get smaller. However, whether one measures airway resistance, forced expiratory volume in 1 s, airway areas, or any other indexes, there are at least three ways to quantify the level of contraction. One can calculate the absolute change from baseline, but the more common way is to calculate this change as a fraction or percentage of the baseline. This method implicitly assumes that there are no substantive changes in the baseline. In our studies, however, we know that there are in fact quite large temporal changes in baseline size, so we have commonly presented airway responses as fractions of the completely relaxed airway size. However, if one imagines that a given dose of MCh should lead to a similar constricted size, then perhaps the absolute size at a given dose should also be an appropriate metric. In the end, without knowing what causes the baseline variability, it is not possible to know what the best metric is to assess airway responsiveness.

In the present study, we have, therefore, not limited ourselves to one metric but rather present the absolute sizes with the different doses as well as the percentages of relaxed size. Although we knew from prior work that airways were variable at baseline, we had little intuition as to what would happen with exogenous contraction. If an airway was less contracted at baseline on one day, would it contract more or less than on another day when it had more baseline tone? Since a fully relaxed airway has the whole range of size (down to closure) to narrow, one might superficially expect it to have greater variability than one that is already narrowed at baseline (where the range would be that much smaller). Furthermore, since the whole range of sizes during contraction is smaller than at baseline, one might also have expected there to be less variability with contraction than that at baseline. Our results, however, do not support this reasoning.

Airways with any level of MCh stimulation had greater variability than those with no exogenous MCh. The airway variability was greatest at the lowest dose of MCh administered but was elevated at all the doses. These data demonstrate that for individual airways, there was significant response variability even when the same dose was administered on subsequent days. We were surprised to find no relationship between the level of activation and the CV. In part, this resulted from the fact that our lowest dose caused such a large average airway constriction. Furthermore, with only four data points per airway to calculate the standard deviation and thus the CV, we did not have sufficient sensitivity with the relatively small average difference between the three doses to make a further inference.

It was interesting to observe the range of the responses among the population of airways. For a few airways, there was little or no variability to their MCh responses over the 4 days measured ($CV = 0$). For most other airways, however, there was wide variability both between and within dogs in the response to a given dose of MCh ($CV = 33\text{--}38\%$). Although we saw significant differences in the mean variability between dogs (Tables 1 and 2), we also saw significant individual airway variability within each dog. Since we were infusing the MCh at a steady-state constant concentration, the variability must be related to intrinsic differences at the individual airway level.

As the measure of variability, for each dog at each airway site, at baseline and each dose of MCh, we calculated the CV from the four measurements made on different days. The CV is calculated as the standard deviation divided by the mean of the measurements. We felt the choice of CV was the appropriate statistical metric, since the CV includes normalization by the mean size.

Experimental methods

To minimize any potential variability associated with the methods, all dogs were anesthetized, intubated, and ventilated in the same manner at the same time of day on the four occasions. Furthermore, to avoid a varying time course associated with an acute aerosol challenge, we administered the spasmogen as a continuous intravenous administration that, in the absence of vascular disease, should have reached all the airways with the same concentration. In addition, we waited 20 min after beginning each dose of the continuous infusion, about four half-lives longer than the response time for the airways to MCh, to assure we were at steady state before acquiring the scans. Locating the same airway on different days was straightforward and has been documented in several previous studies (4–7, 9, 11, 12).

The intervals between the repeated studies ranged from 1 to 8 wk and were based on HRCT scanner availability. Although we strived to maintain comparable intervals between the studies, because of scheduling constraints associated with the clinical CT scanner, it was not

always possible. Although constant intervals between each study session would have been preferred, there were no indications that the time interval between measurements had any consistent effect on our results. This is consistent with previous work from our laboratory on baseline sizes (14), where the time interval between the baseline studies also varied widely, with no correlation between the length of time and the extent of baseline tone. What we still do not know are the limits of this time frame of variability. How much of this variability is similar over shorter intervals of hours or days remains to be determined.

To optimize the measurement of airway area, all measurements were made by the same person (K. Fallano). We also only measured airways with a baseline diameter >2 mm in diameter, which has been shown to be a size above which there is sufficient signal to noise and limited measurement variability (29). In addition, all the measurements were made at functional residual capacity, so lung volumes within a dog should not have been different.

Literature studies relevant to airway heterogeneity

In recent years, there has been considerable discussion of the heterogeneity of airway responses and the potential role that this may play in health and disease (2, 20, 21, 23, 25, 26, 28, 29, 31, 32, 37–43). Several of these studies have used HRCT or PET imaging to assess the heterogeneity of airway narrowing in individual subjects. Using HRCT, Brown et al. (3) examined the degree of heterogeneity in airway responses to aerosol versus intravenous administration of histamine. They found that there was no statistical difference in the mean level of heterogeneity of the response by either route, suggesting that the route of administration was not a major determinant of airway response heterogeneity. These data showed that there is intrinsic heterogeneity in the overall response of the airways, although the responses of individual airways with each challenge were not compared. Our current data extend those findings by demonstrating that not only was there no change in the variability in the airway response to increasing doses of spasmogen but there was also variability in the day-to-day repeated responses for the same airway given the same doses of spasmogen.

King et al. (29) used HRCT to measure the airways in healthy and asthmatic individuals. The subjects were challenged with MCh, and King et al. measured the variability in airway responses. They found that the degree of heterogeneity of airway narrowing on a single day challenge in asthmatic subjects was greater than that in the healthy subjects (29). Although they also performed repeated baseline scans on a subset of their subjects, this was only done on the healthy and not the asthmatic subjects. Although the heterogeneity they observed in responsiveness is similar to what we find in the dogs on a single day, since they only examined responsiveness on one occasion, it is not possible to compare our findings on multiple days. One would like to speculate that the variability is greater in the asthmatic subjects, perhaps leading to episodic airway narrowing, but until repeated studies are done, this will remain uncertain.

With the use of PET scanning combined with computational modeling, it has been shown that heterogeneity in airway response to a bronchoconstrictor is necessary to explain the patchy ventilation observed during an induced asthma attack (37, 38, 41, 43). On PET scans, one can observe organized areas of patchy nonventilated lung regions. To explain these patchy areas, Venegas et al. (41) used a mathematical model to show that, even for uniform smooth muscle activation of a symmetric bronchial tree, the presence of very minimal heterogeneity breaks the symmetry and leads to large clusters of poorly ventilated lung units. Our present data are consistent with this notion and strongly support this modeling of response heterogeneity. Of perhaps even more interest and relevance to the Venegas model (41), we found that the heterogeneity was not constant for a given airway. Thus, in their model, every time constriction occurs, the airway response heterogeneity could occur from a

different set of airways, leading to different clusters of poorly ventilated lung units. If such heterogeneity in response to a spasmogen was greater in individuals with asthma compared with healthy individuals, then it might possibly lead to unpredictable airway narrowing and the pathological patchiness observed clinically.

Fredberg et al. (18) used alveolar capsules in dogs to assess the heterogeneity of lung responses to aerosolized histamine. Their method enabled the measurement of multiple pressures in many peripheral lung regions. Based on the variability in regional pressures, they inferred the behavior of airways supplying those regions to conclude that there was marked regional heterogeneity in airway responses to aerosol histamine.

Another method of estimating global heterogeneity in the lung is with lung impedance (20, 21, 28, 33). The degree of heterogeneity is based on modeling the changes in the frequency dependence of lung resistance and elastance. With this approach, it has been shown that the heterogeneity was higher in subjects with more severe asthma compared with subjects with mild to moderate asthma and healthy controls (27, 33). The reproducibility of this assessment was not determined, and the method obviously can provide no insight into the spatial or anatomic details underlying the heterogeneity. However, these models can improve estimates regarding which diameters are involved in producing the heterogeneity of ventilation when combined with imaging techniques (38). A few studies have been performed in children using impedance measurements, and their findings support our results. In clinically stable asthmatic children, Goldman et al. (22) demonstrated day-to-day variability in respiratory impedance not detected by spirometry. Consistent with our findings while examining airway relaxation rather than stimulation, Lall et al. (30) also demonstrated that the variability in the respiratory resistance decreased after the administration of bronchodilators in children with asthma.

Possible explanations of observed variability

At the present time, we have no definitive explanation of the observed variability in both baseline size and the responses to the same doses of MCh. There are several concepts, however, that lead to some speculation about the underlying causes of the variability. Remodeling and changes in airway smooth muscle mass have been shown to exist in asthmatic individuals (16, 17, 35). Although differences in smooth muscle mass may explain baseline and response differences among airways, they could not explain the day-to-day differences we observed in the same airways of healthy dogs. Similarly, any potential differences in muscarinic receptor density could not explain the day-to-day response variability of an individual airway. Variations in local vagal tone surely could lead to variability in the baseline airway size, but there is no information on either the spatial distribution of vagal tone to the airways or its temporal variation. Similar temporal and spatial ignorance exists with regard to variations in the local milieu bathing the airways. It thus seems that perhaps the airway smooth muscle itself is responsible for both the widely varying baseline tone and the variable response to exogenous stimulation. If so, then it raises questions about whether there is any possible function of this variation or whether it is just another manifestation of the otherwise useless nature of airway smooth muscle (34). The impact of the variability on lung pathology also remains to be determined. If one examines the variation in spirometric measures of lung function in humans with normal and diseased lungs over time, one finds that it is quite reproducible (15, 36). Such measures, of course, are only indirect measures of airway size and, even then, reflect the composite average of tens of thousands of parallel and series airways. In a study by Frey et al. (19) using a fractal model, they observed that increased variability in peak expiratory flows was associated with the most severe asthmatics and with an increased risk of unstable airway function. The potential relevance of the variability in individual airway size and responsiveness could be

resolved with studies that followed individual airway responses over time in asthmatic or normal subjects.

Conclusion

These data confirm the substantial day-to-day variability in baseline airway size in individual airways. In addition, the same dose of MCh to the same individual airways shows considerable variability in responsiveness. The mechanisms underlying this variability are poorly understood, but if we consider that increased heterogeneity may potentiate clinical disease, then we speculate that we would find greater day-to-day airway variability in asthmatic than in healthy individuals.

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Fig. 1.

The diameter (Dia; in mm) for each diameter at baseline and during methacholine (MCh) infusion of 17 (A), 67 (B), and 200 $\mu\text{g}/\text{min}$ (C) for each airway. There were significant correlations of the airway size at baseline and during the MCh infusion for the 17 ($r = 0.93$, $P < 0.00001$), 67 ($r = 0.91$, $P < 0.00001$), and 200 $\mu\text{g}/\text{min}$ ($r = 0.91$, $P < 0.00001$) doses. Even with these high correlations, note the wide range of responsiveness, especially in the middle of the baseline size range, as demonstrated by the vertical spread of airway responsiveness at each dose.

Fig. 2.

The coefficient of variation (CV) for all the airways in all 5 dogs at baseline (MCh 0) and MCh doses of 17, 67, and 200 $\mu\text{g}/\text{min}$. There was significant variability in the CV among the dogs and by dose (see results).

Table 1

The mean CV for each dog for each dose of MCh

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5
MCh (0 baseline), %	12.8	11.0	7.4	3.9	19.8
MCh (17 µg/min), %	13.7	24.5	22.2	15.5	21.2
MCh (67 µg/min), %	16.7	11.7	21.8	12.6	9.3
MCh (200 µg/min), %	13.0	17.0	16.7	12.1	8.0

CV, coefficient of variation; MCh, methacholine. See article for more details.

Table 2

The P values for the between-dog comparisons of the CV

	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5
<i>Dog 1</i>					
<i>Dog 2</i>	0.0009				
<i>Dog 3</i>	<0.0001	0.07			
<i>Dog 4</i>	<0.0001	<0.00001	<0.00001		
<i>Dog 5</i>	0.09	0.07	0.0004	<0.0001	