



Comparison of direct and indirect bronchoprovocation testing using ventilator-acquired pulmonary mechanics in healthy cats and cats with experimental allergic asthma



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ABSTRACT

Airway hyperresponsiveness (AHR) is a key feature of asthma and can be measured using bronchoprovocation. Direct (methacholine, MCh) or indirect (adenosine-5-monophosphate, AMP; or mannitol) bronchoprovocants are used in human patients, the latter inducing AHR only with pre-existing airway inflammation. The present study compared the responses to direct (MCh) and indirect (mannitol, AMP) bronchoprovocation in healthy and asthmatic cats ($n = 6/\text{group}$). The order of bronchoprovocant was randomized using a published table of random numbers and there was a 1-month washout before cross-over to the next treatment. Pulmonary mechanics were measured in anesthetized and mechanically ventilated cats using a critical care ventilator. Saline at baseline and increasing doses of each bronchoprovocant were aerosolized for 30 s, followed by 4 min of data collection between doses. The endpoint for each bronchoprovocant was reached when airway resistance exceeded 200% of baseline values (EC200Raw).

There was a significant difference ($P < 0.001$) in the airway response of asthmatic vs. healthy cats over the range of MCh concentrations, despite there being no significant difference in the EC200Raw between the groups. Response to MCh was significantly greater ($P < 0.05$) in asthmatic than in healthy cats at MCh concentrations as low as 0.0625 mg/mL. For AMP, a small subset of asthmatics ($n = 2/6$) responded at low concentrations; four asthmatic cats and all healthy cats failed to respond even to the highest concentrations of AMP. One asthmatic cat but no healthy cats responded to mannitol. In conclusion, MCh discriminated asthmatic from healthy cats but neither AMP nor mannitol was an effective bronchoprovocant in this model.

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Introduction

Feline asthma is an inflammatory lower airway disease that leads to wheezing, coughing, respiratory distress or even death. Three important hallmark features, airway hyperresponsiveness (AHR), eosinophilic airway inflammation, and airway remodeling, contribute to these clinical signs. AHR, defined as narrowing of airways too much or too easily in response to a stimulus (Scichilone et al., 2006), can be detected in cats by the administration of a bronchoprovocant, followed by measuring changes in airway resistance (Norris Reinero et al., 2004; Lee-Fowler et al., 2012). Measuring AHR has been used to diagnose disease, evaluate disease severity, monitor treatment efficacy, and adjust drug dosages in human asthmatics (Sont et al., 1999; O'Byrne, 2010; Brannan and Loughheed, 2012).

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In cats, airflow limitation has been estimated by tidal breathing flow-volume loops (McKiernan et al., 1993), forced expiratory flow-volume curves using thoracic compression (Bark et al., 2007), or barometric whole body plethysmography (BWBP; Hoffman et al., 1999; Kirschvink, 2008; Hirt et al., 2011). While minimally invasive, these techniques do not provide accurate direct measures of airway resistance, which is preferred for functional evaluation (Bates et al., 2004). Our laboratory uses ventilator-acquired pulmonary mechanics in anesthetized and intubated healthy and experimentally induced asthmatic cats to obtain a consistent and reliable measure of airway resistance (Lee-Fowler et al., 2009, 2012).

Methacholine (MCh) has traditionally been used as a direct bronchoprovocant to facilitate the measurement of AHR in both humans and experimentally induced asthmatic cats (Norris Reinero et al., 2004; Brannan, 2010; Lee-Fowler et al., 2012). Direct bronchoprovocants stimulate airway constriction by interaction with specific airway smooth muscle receptors (Cockcroft and Davis, 2006) such as muscarinic (MCh) or histaminic receptors

(histamine). Studies have found dissociation between the severity of airway inflammation and functional airway responses with direct bronchoprovocants including MCh and histamine (Crimi et al., 1998; Rosi et al., 1999).

The airway response to direct bronchoprovocants has variable components (reflecting the inflammatory aspect of AHR) and fixed components (reflecting airway remodeling; Brannan and Loughheed, 2012). In any asthmatic, there can be different degrees of each of these components. Additionally, other airway disorders can have variable and fixed components of the airway response to direct bronchoprovocants. Studies in human asthmatics have shown direct bronchoprovocation testing has a high sensitivity but a low specificity for asthma (Woolcock and Peat, 1989; Cockcroft, 2010).

In performing MCh challenges many times in healthy and experimentally asthmatic cats, we have noted that the response to MCh as assessed by the EC200Raw¹ is repeatable, as is the effective concentration of MCh required to increase baseline airway resistance by 200%, in the same cat tested on different days (Lee-Fowler et al., 2009). However, we have also noted overlap between responses of asthmatic and healthy cats. Moreover, some asthmatic cats require large doses of MCh to reach the EC200Raw despite airway eosinophilia and allergen sensitivity (unpublished data). This observation has stimulated interest in assessing indirect bronchoprovocants in cats.

Indirect bronchoprovocants such as adenosine-5-monophosphate (AMP) or mannitol have been employed in humans to assess AHR, and compared with MCh, are highly specific but have a relatively low sensitivity (Sverrild et al., 2010). They cause airway narrowing by releasing contractile mediators (e.g. histamine, prostaglandins, and leukotrienes) from existing inflammatory cells such as eosinophils or mast cells within airways (Joos et al., 2003), which subsequently interact with specific receptors on airway smooth muscle cells. AHR to indirect bronchoprovocants appears to be dependent on the number of airway inflammatory cells (Duong et al., 2008; Porsbjerg et al., 2008). Additionally, indirect bronchoprovocants might work on activated sensory neural pathways in asthmatic patients (Anderson, 2010). Thus, indirect bronchoprovocation tests could more readily discriminate asthmatic cats from healthy cats.

While MCh is considered the reference standard in experimental feline asthma for direct measurement of AHR, AMP and mannitol have not been evaluated in this context. Additionally, while bronchoprovocation with AMP appears to hold promise for discriminating healthy from bronchitic cats using barometric whole body plethysmography (Hirt et al., 2011), the respiratory value derived from this technique (the enhanced pause) is not equivalent to airway resistance. No published study to date has assessed direct measures of airway resistance comparing MCh, AMP, and mannitol in healthy and asthmatic cats. We hypothesized that the indirect bronchoprovocants AMP and mannitol would be superior in discriminating experimentally asthmatic cats from healthy cats compared to the direct bronchoprovocant MCh using ventilator-acquired-pulmonary mechanics.

Materials and methods

Animals

Twelve male research cats (1–2 years old) were enrolled and cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals, with approval by the University of Missouri Animal Care and Use Committee (ACUC protocol 6912). Six healthy cats were used as controls (airway lavage confirmed lack of inflammation prior to study enrollment) and the other six cats were experimentally induced to have asthma using Bermuda grass allergen (BGA).

Asthma induction

Six naïve cats were sensitized with BGA as previously described (Norris Reinero et al., 2004) with minor modifications; cats received weekly aerosol challenges with BGA until study completion. In brief, on day 0, cats received a SC injection of 12 µg of BGA in 10 mg of alum and 100 ng *Bordetella pertussis* toxin SC; on day 14, intranasal BGA (75 µg of BGA in 200 µL of PBS); and on day 21, 12 µg of BGA in 10 mg alum SC. On day 28, the formation of wheals on an intradermal skin test confirmed sensitization to BGA. For the following 2 weeks, intensive aerosol challenges were administered to all cats with BGA (500 µg of BGA in 4 mL PBS) over 10 min. On day 42, bronchoalveolar lavage fluid (BALF) was collected and the asthmatic phenotype was confirmed (defined as >17% eosinophils). Cats received weekly aerosol challenges of BGA for 3 months prior to study enrollment.

Animal and ventilator preparation for pulmonary mechanics

Cats were fasted overnight prior to the assessment of airway reactivity. Sedation was administered using ketamine (30 mg IV) and to facilitate placing an IV catheter in a cephalic vein. Anesthetic induction was performed using 6 mg/kg of propofol IV. Cats were intubated with a 4 mm internal diameter, cuffed endotracheal tube that was cut to a standardized length of 140 mm. The same size and length of endotracheal tube was used in all cats to standardize any resistance from the endotracheal tube. A continuous infusion of propofol (0.3 mg/kg/min) was used to maintain anesthesia and intermittent boluses of propofol (0.5–1 mg/kg) were given whenever evidence of spontaneous breathing effort was apparent. Cats were connected to a ventilator (GE Engstrom Carestation) operated in neonatal mode.

A 5 French gauge tracheal catheter (Cardinal Health) was inserted to the distal end of the endotracheal tube to measure airway pressure and a heated pneumotachograph was used to measure gas flow. The ventilator was operated in volume-control mode and ventilation parameters set as follows: tidal volume 10 mL/kg; respiratory rate 10 breaths/min; inspired oxygen fraction 0.4; inspiratory-to-expiratory ratio 1:3, and positive end expiratory pressure (PEEP) 5 cm H₂O. The ventilator was checked for appropriate function and calibration each day of the pulmonary function test. Respiratory parameters were adjusted to maintain an end-tidal CO₂ of 34–40 mmHg.

Prior to baseline data collection, cats were ventilated for 5 min to reach a steady state. Sterile 0.9% saline was delivered for 30 s through an Aeroneb solo in-line nebulizer (Aeroneb Pro; Aerogen) located in the inspiratory limb of the anesthetic circuit, followed by 4 min of baseline data collection. The nebulizer produced particles having a mass median aerodynamic diameter of 2.1 µm. The pneumotachograph was transiently disconnected during nebulization (30 s) to protect the instrument from particulate matter. Airway resistance (Raw) and compliance were constantly calculated by the ventilator and recorded by the data collection system (E-view, Datex-Ohmeda). Hemoglobin saturation and pulse rate were continuously monitored using pulse oximetry and recorded every 60 s during data collection as a safety measure. End-tidal carbon dioxide and body temperature were recorded just prior to the administration of agonists and after the completion of the experiment.

Bronchoprovocation test

All cats received MCh, AMP and mannitol in random order using a published table of random numbers (Daniel, 1999). There was a 1-month washout period between each bronchoprovocant. Bronchoprovocation testing was performed by delivering doubling doses of each bronchoprovocant (MCh, 0.0625–32 mg/mL; AMP, 0.39–200 mg/mL; mannitol, 5–160 mg/mL) through the in-line nebulizer for 30 s followed by 4 min of data collection. The highest concentration of mannitol (160 mg/mL) was repeated a total of three times. Bronchoprovocation testing was terminated when Raw increased 200% over baseline (i.e. after aerosol saline). Data were expressed as the concentration of each bronchoprovocant that increased Raw by 200% over baseline (EC200Raw; the maximum Raw obtained over the 4 min of data collection at each bronchoprovocant dose was used for this calculation) or airway resistance (cmH₂O/L/s) after 2 min in response to each concentration of each bronchoprovocant. The EC200Raw was calculated by linear interpolation of the log plot of the dose response curve with the response expressed as the percentage of baseline Raw. A fast acting β-2 agonist (albuterol) was administered when clinically indicated after the bronchoprovocation test ended.

Bronchoalveolar lavage fluid collection and analysis

After bronchoprovocation testing, BALF was collected using a blind technique (Reinero et al., 2011a). A cytocentrifugation preparation was stained with a modified Wright's stain set for microscopic evaluation. A 200 cell differential count was performed and the percentage of eosinophils was recorded.

Statistical analysis

Comparisons of the mean EC200Raw of MCh between the two groups (healthy vs. asthmatic cats) were made using *t* tests and if the EC200Raw was not achieved with 32 mg/kg MCh, the value was set at 64 mg/kg for analysis (Van Den Berge

¹ EC200Raw is the endpoint for each bronchoprovocant reached when airway resistance exceeds 200% of baseline values.

et al., 2001; Currie et al., 2003b). Two-way repeated measures ANOVA testing was performed to evaluate the difference in EC200Raw between the healthy and asthmatic groups for MCh, AMP and mannitol. Post hoc analysis was performed using the Holm–Sidak method to determine differences between groups at individual concentrations of each agonist. Statistical analysis was limited to concentrations between 0 and 2 mg/mL of MCh, 0 and 3.125 mg/mL of AMP, and 0 and 10 mg/mL of mannitol (i.e. complete data sets above 2 mg/mL of MCh, 3.125 mg/mL of AMP, and 10 mg/mL of mannitol data were not available because the bronchoprovocation study was terminated in reacting cats). $P < 0.05$ was considered statistically significant.

Results

Methacholine

There was no significant difference in the group mean EC200Raw between healthy and asthmatic cats ($P = 0.329$; Table 1). However, while the group mean ‘endpoint’ of the study did not differ, there was a significant difference between groups in airway resistance over the range of MCh concentrations ($P = 0.005$; Fig. 1). This accentuated response to MCh was detected in asthmatic cats at a concentration as low as 0.0625 mg/mL and up to 2 mg/mL. Also, when the airway resistance at each concentration of MCh was compared to airway resistance after saline aerosol within each group (i.e. baseline resistance), three concentrations of MCh (0.5, 1.0 and 2.0 mg/mL) showed significant increases in asthmatic but not healthy cats ($P < 0.001$). The airway resistances of each cat in both groups in response to the entire range of MCh concentrations are shown in Fig. 2a.

AMP

Using AMP as the bronchoprovocant, a statistically significant difference between groups was detected at 3.125 and 6.25 mg/mL ($p = 0.005$ and $p = 0.010$, respectively). However, four asthmatic and all six healthy cats failed to reach the EC200Raw even with the highest concentration of AMP (Fig. 2b). The percentage of eosinophils in BALF of asthmatic cats at the time of AMP challenge was 48 ± 19 (mean $\pm$ standard deviation, SD).

Mannitol

Only one asthmatic cat reached the EC200Raw using mannitol as a bronchoprovocant and that was at a concentration of 10 mg/mL. Five asthmatic and all six healthy cats failed to reach a 200% increase of airway resistance over the baseline, even with the highest concentration of mannitol (Fig. 2c). No statistically significant difference was found between the healthy and asthmatic groups at any concentration of mannitol when airway resistance was compared between two groups. The percentage of eosinophils in BALF of asthmatic cats when challenged with mannitol was 61 ± 14 (mean $\pm$ SD).

Table 1

Percentage eosinophils in BALF and the concentration of methacholine (mg/mL) that increased airway resistance by 200% over baseline (EC200Raw) in healthy and asthmatic cats. There was no significant difference in the group mean EC200Raw between healthy and asthmatic cats ($P = 0.329$).

Cat	Phenotype	Eosinophils (%)	EC200Raw (mg/mL)	Cat	Phenotype	Eosinophils (%)	EC200Raw (mg/mL)
1	Healthy	5	19.0	7 ^a	Asthmatic	65	2.0
2	Healthy	7	8.9	8	Asthmatic	56	4.1
3	Healthy	2	13.4	9 ^a	Asthmatic	87	9.7
4	Healthy	5	3.8	10	Asthmatic	65	32.0
5	Healthy	10	8.9	11	Asthmatic	73	23.5
6	Healthy	3	7.8	12 ^b	Asthmatic	52	5.5
	Mean $\pm$ SD	5 $\pm$ 3	10.3 $\pm$ 5.2		Mean $\pm$ SD	66 $\pm$ 13	12.8 $\pm$ 12.2

SD, standard deviation.

^a Responded to AMP.

^b Responded to mannitol.

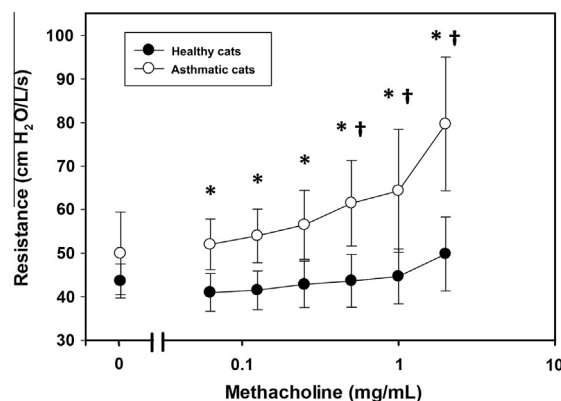


Fig. 1. The mean and standard deviation of airway resistance in response to MCh challenge (baseline and doubling doses between 0.0625 mg/mL and 2 mg/mL) in healthy (black circle) and asthmatic (white circle) groups documents significant differences in MCh bronchoprovocation between groups. Asthmatic cats had significant increases in airway resistance compared to healthy cats at every dose of MCh between 0.0625 mg/mL and 2 mg/mL. Asthmatic cats but not healthy cats also had significant increases in airway resistance at MCh doses of 0.5, 1.0 and 2.0 mg/mL compared with baseline (saline). * $P < 0.05$ versus healthy group † $P < 0.05$ versus baseline airway resistance.

Discussion

The results of this study showed that, in contrast to our hypothesis, indirect bronchoprovocants were not effective in discriminating asthmatic from healthy cats and only the direct bronchoprovocant MCh was able to show a differential response between groups. Although indirect bronchoprovocants lead to AHR by stimulating airway inflammatory cells to release various inflammatory mediators (Van Den Berge et al., 2001; Porsbjerg et al., 2008; Brannan, 2010), the indirect bronchoprovocants did not induce a reliable airway response in asthmatic cats. No healthy cat responded to even the highest concentration of AMP or mannitol. In contrast, asthmatic cats had significant increases in airway resistance after MCh challenge over the range of MCh concentrations compared to healthy cats. This occurred despite no significant difference between groups with MCh challenge in EC200Raw, the traditional measure of AHR.

Methacholine is the most common bronchoprovocant used in human asthmatics and is considered a highly sensitive bronchoprovocant for functional airway testing. It is best used to exclude a diagnosis of current asthma in symptomatic patients that are able to inhale high doses of MCh without reacting (i.e. reaching the endpoint of the test, which is a 20% decrease in the forced expiratory volume in 1 s); however, in patients who react at very low doses, it is also highly specific for asthma (Cockcroft, 2010).

There is a gray zone of interpretation when patients respond to intermediate doses of MCh, which is in large part why indirect

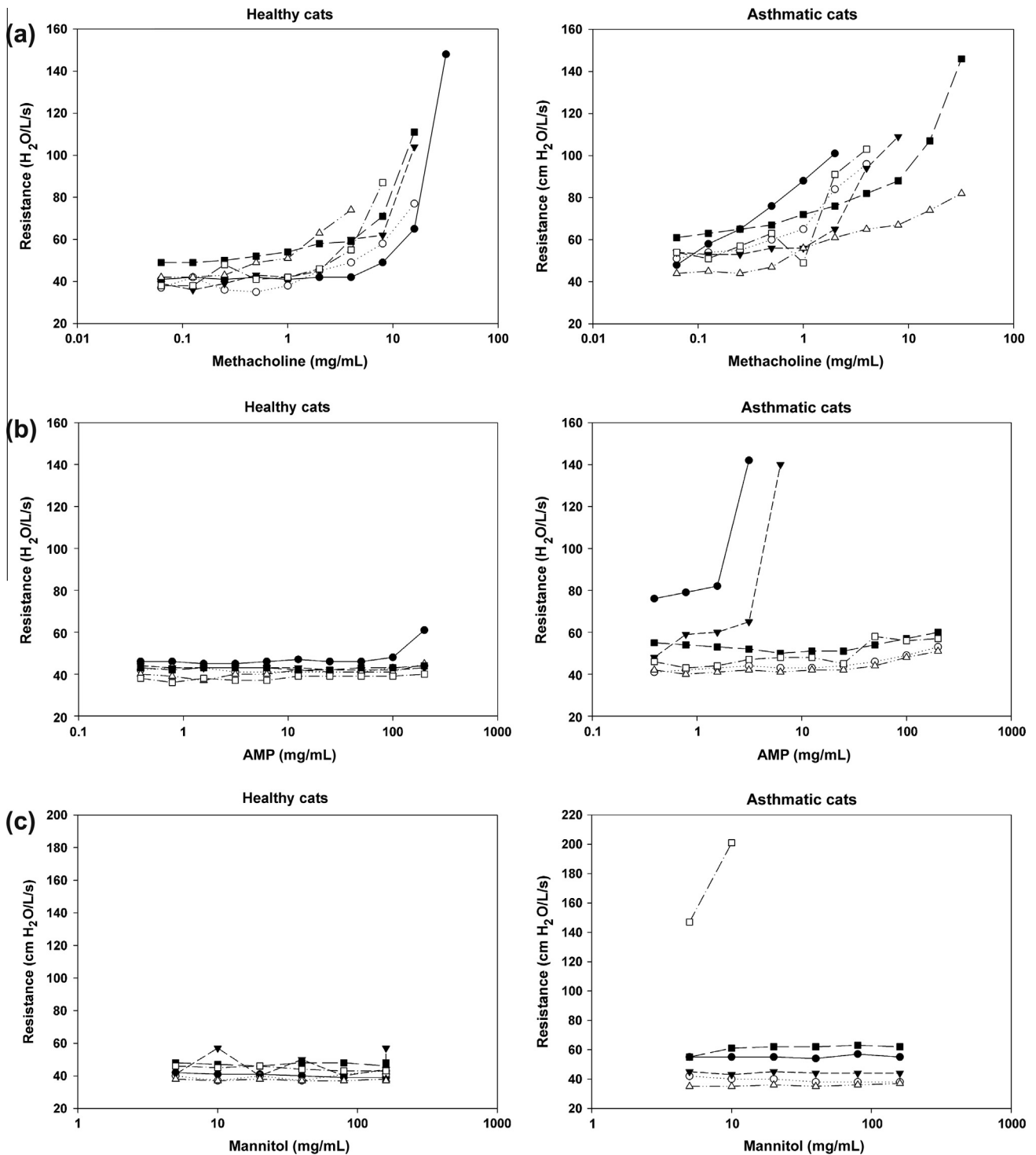


Fig. 2. The highest airway resistance of each cat in both groups (healthy and asthmatic; $n = 6/\text{group}$) in response to each concentration of MCh, AMP and mannitol is displayed in (a, b, and c), respectively. The five healthy and four asthmatic cats in MCh (a), two asthmatic cats in AMP (b), and one asthmatic cat in mannitol (c) that reached EC200Raw before the highest concentration of each bronchoprovocant have missing data after the concentration where they reached EC200Raw, as the endpoint of the study had been reached.

bronchoprovocants have been investigated for their utility. In our feline model, after having performed MCh challenges many times, we have noticed some degree of overlap in the endpoint of the MCh challenge (EC200Raw) between healthy and asthmatic cats over midrange doses (unpublished data). This observation led to the current investigation in the hope that indirect bronchoprovocants would consistently demonstrate AHR only in asthmatic cats

with pre-existing airway inflammation. It is of interest that in general, airway resistance in response to MCh in healthy cats seemed to plateau at low doses and then responded sharply at higher concentrations of MCh (Fig. 1). In contrast, asthmatic cats tended to initiate airway constriction at lower concentrations of MCh. However, we were unable to determine a cut-off concentration that clearly differentiated between asthmatic and healthy cats.

As an indirect bronchoprovocant, AMP is believed to cause AHR by releasing mediators through activation of A2 receptors on airway mast cells (Polosa et al., 2002). In human asthmatics, bronchoprovocation using AMP was more closely correlated with the severity of eosinophilic inflammation and the concentration of AMP required to cause AHR when compared to bronchoprovocation using MCh (Van Den Berge et al., 2001). In a similar fashion, a previous study evaluating AMP as a bronchoprovocant in pet cats with asthma, pet cats with chronic bronchitis, and healthy research cats, showed that AMP challenge led to airflow limitation in the majority of cats with lower airway disease and produced no response in healthy cats (Hirt et al., 2011). However, that study used barometric whole body plethysmography (BWBP) to determine the enhanced pause (Penh), a surrogate marker of airflow obstruction that has been criticized for not reflecting airway resistance (Bates et al., 2004; Kirschvink, 2008). The present study evaluated AHR by directly calculating airway resistance using ventilator-acquired pulmonary mechanics, which enabled the delivery of each bronchoprovocant directly to the airways from the nebulizer via the endotracheal tube.

Results of published studies of BWBP and ventilator-acquired pulmonary mechanics in cats have been somewhat discordant, perhaps because they do not measure the same outcome parameter (i.e. BWBP measures Penh, whereas ventilator-acquired pulmonary mechanics directly measures airway resistance). This is true for AMP bronchoprovocation, as in one published study, 9/15 pet cats with spontaneous lower airway disease reached the endpoint of BWBP testing (Hirt et al., 2011), whereas the endpoint was reached in 2/6 cats with experimentally induced asthma in the current study. However, it is of note that none of the healthy cats in either study reacted to AMP. While these results might make it tempting to speculate that BWBP is more sensitive than ventilator-acquired pulmonary mechanics, results of other studies refute that. Specifically, discordant results were noted when testing was conducted to determine whether aerosolized N-acetylcysteine causes bronchospasm when inhaled (Shibly and Hirt (2009) and Reinero et al., 2011b).

While measurements taken using BWBP appeared to show that N-acetylcysteine was safe in cats, producing no significant changes in Penh (Shibly and Hirt, 2009), ventilator-acquired pulmonary mechanics documented that every cat had increases in baseline resistance (group average 150% increase in baseline resistance) and at least one adverse reaction (Reinero et al., 2011b). Published feline studies to date have not compared BWBP and ventilator acquired pulmonary mechanics in a head-to-head fashion in the same cats with the same bronchoprovocant.

These are important factors in evaluating the sensitivity, advantages and limitations of each method and determining the correlation of AHR with other parameters such as airway inflammation or airway remodeling. In the current study, AMP bronchoprovocation was specific, showing AHR only in experimentally induced asthmatic cats and not in healthy cats. However, it was poorly sensitive, failing to document AHR in four asthmatic cats. It is possible that higher doses of AMP could have led to a response where the resistance curves drifted up from baseline at the higher doses in asthmatic cats, such as in Fig. 2b.

Inhaled mannitol is believed to cause AHR by inducing extracellular airway fluid to become hyperosmolar, thus stimulating inflammatory cells to release a variety of mediators (Anderson et al., 1997; Brannan et al., 1998). Because this mechanism depends on airway inflammation to cause AHR, a positive response to mannitol indicates the presence of airway inflammation (Porsbjerg et al., 2008) and also implies healthy individuals should not respond to mannitol bronchoprovocation. Consequently, inhaled mannitol has been used to diagnose or to monitor the response

of treatment in human asthma (Brannan et al., 1998; Anderson, 2008; Brannan, 2010).

To the authors' knowledge, the present study is the first to evaluate mannitol as a bronchoprovocant in asthmatic cats. Although the dose of mannitol used here was extrapolated from human studies (Currie et al., 2003a), it is possible that a higher concentration might have evoked a response in the asthmatic cats. Also, the formulation of mannitol was a liquid rather than a dry powered inhalant, as is used in humans (Brannan et al., 1998; Currie et al., 2003a; Anderson, 2008). The dry powder formulation requires a conscious effort and the ability to inhale maximally on command. This maneuver is not possible in cats. Finally, asthmatic cats might have different intracellular events in response to increased osmolality induced by mannitol compared with human asthmatics. Regardless, mannitol as used in this study does not appear to be a suitable bronchoprovocant for asthmatic cats because of its low sensitivity.

This study had some limitations. First, the cats we used were experimentally induced to have asthma and they represent a model of spontaneous disease. Further studies are warranted evaluating the use of bronchoprovocants with direct measures of airway resistance in pet cats with spontaneous asthma. Additionally, because of the heterogeneous asthmatic phenotype, larger numbers of cats should be tested to determine if stratifying results based on asthma severity at enrollment might improve the sensitivity of indirect bronchoprovocation testing. Finally, future studies could focus on using higher doses of indirect bronchoprovocants to investigate whether these doses might be more useful to discriminate asthmatic from healthy cats.

Conclusions

Bronchoprovocation using MCh discriminated between experimentally induced asthmatic cats and healthy cats, based on differential responses in airway resistance over the range of concentrations nebulized. Unexpectedly, despite a clear-cut difference in airway eosinophilia between healthy and experimentally induced asthmatic cats, very few asthmatic cats responded to either AMP or mannitol. However, since none of the healthy cats responded to these indirect bronchoprovocants, there is support for the premise that AHR is induced is by activating inflammatory cells accumulated in the airways.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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