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Methacholine-induced pulmonary gas trapping in a mouse model of allergic asthma: Effect of inhaled budesonide and ciglitazone

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Abstract

Previously, we found pulmonary gas trapping to be a rapid, simple and objective measure of methacholine-induced airway obstruction in naïve mice. In this study we extended that finding by using methacholine-induced pulmonary gas trapping to differentiate airway responses of ovalbumin-sensitized, ovalbumin-exposed (Positive Control) and ovalbumin-sensitized, sodium chloride-exposed (Negative Control) mice. Additionally, pulmonary gas trapping and enhanced pause were compared following methacholine exposure in sensitized and nonsensitized mice. Finally, we examined by nose-only inhalation the ability of the glucocorticosteroid budesonide and the peroxisome proliferator-activated receptor-γ agonist ciglitazone to modify methacholine-induced airway responses in ovalbumin-sensitized mice. Positive Controls exhibited a 7.8-fold increase in sensitivity and a 2.4-fold enhancement in the maximal airway obstruction to methacholine versus Negative Controls. Following methacholine, individual Positive and Negative Control mouse enhanced pause values overlapped in 9 of 9 studies, whereas individual Positive and Negative Control mouse excised lung gas volume values overlapped in only 1 of 9 studies, and log[excised lung gas volume] correlated ($P=0.023$) with in vivo log[enhanced pause] in nonsensitized mice. Finally, budesonide (100.0 or 1000.0 μg/kg) reduced methacholine-mediated airway responses and eosinophils and neutrophils, whereas ciglitazone (1000.0 μg/kg) had no effect on methacholine-induced pulmonary gas trapping, but reduced eosinophils. In conclusion, pulmonary gas trapping is a more reproducible measure of methacholine-mediated airway responses in ovalbumin-sensitized mice than enhanced pause. Also, excised lung gas volume changes can be used to monitor drug interventions like budesonide. Finally, this study highlights the importance of running a positive comparator when examining novel treatments like ciglitazone.

Keywords: Asthma; Airway inflammation; Airway obstruction; Airway resistance; Inhaled glucocorticosteroids; Peroxisome proliferator-activated receptor-γ agonist

1. Introduction

Airway narrowing is a result of increasing bronchomotor tone (Orehek, 1980) such as that caused by methacholine acting at muscarinic M_3 receptors located on airway smooth muscle (Stengel et al., 2002). Excessive bronchoconstriction to methacholine is a prominent feature of human asthma (Gibbons et al., 1996), and leads to pulmonary hyperinflation as indicated by increased amounts of trapped air behind occluded airways (Lovejoy et al., 1961; Woolcock and Read, 1985). In previous studies, we have shown increases in pulmonary gas trapping in guinea pigs correlate highly with bronchoconstrictive agonist-mediated decreases in dynamic compliance and increases in total pulmonary resistance and is predictive of the state of in vivo airway obstruction at death (Stengel et al., 1987; Stengel and Silbaugh, 1989). More recently, we found dose-related excised lung gas volumes changes occurred following methacholine bronchoprovocation in mice, although mice were less sensitive than guinea pigs to the airway obstructive effects of methacholine (Stengel et al., 1995). Further, the rank order of sensitivity to methacholine in naïve male A/J, BALB/c and C3H/HeJ mice using pulmonary gas trapping (Stengel et al., 1995; Yiamouyiannis et al., 1995) was similar to that observed in the same mouse strains using a more conventional outcome indicator such as airway pressure–time index to measure acetylcholine-induced airway responsiveness (Levitt and Mitzner, 1989).

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Hammelmann et al. (1997) were the first to describe the use of unrestrained whole-body barometric plethysmography for measuring changes in airway responsiveness to methacholine noninvasively in allergic BALB/c mice. These investigators reported that changes in enhanced pause values after methacholine challenge correlated with changes in intrapleural or esophageal pressure, suggesting that whole-body barometric plethysmography could be used as a measure of bronchoconstriction. Further, they demonstrated that increases in in vivo airway resistance correlated with increases in enhanced pause values determined in the same animals the previous day. Other investigators comparing noninvasive and invasive measurements in allergic C57BL/6 mice have suggested that the relationship between whole-body barometric plethysmography indices and airway resistance for measuring peak airway hyperresponsiveness to methacholine in these mice was inconsistent (Albertine et al., 2002, 2004). However, that enhanced pause correlated with airway resistance in allergic BALB/c mice, suggests a mouse strain-specific effect (Adler et al., 2004).

The mouse model of allergic asthma has been in use for almost 15 years and has provided insight into the pathogenesis of asthma (Epstein, 2004). Although allergic mice do not develop all the characteristic features of human asthma, allergic mice exhibit airway responsiveness to methacholine and eosinophilic pulmonary inflammation (Leong and Huston, 2001; Epstein, 2004). However, airway hyperresponsiveness in mice is an acute phenomenon occurring after allergen challenge, whereas asthmatics demonstrate airway hyperresponsiveness even in the absence of overt disease (Epstein, 2004). Also, the acute airway inflammatory response of mice is markedly different than that of human asthmatics (Kumar and Foster, 2002). For instance, intraepithelial eosinophilic infiltration is observed in human asthmatics, but is less evident in intrapulmonary airways of allergic mice (Korsgren et al., 1997). Thus, when using allergic mice as a model of human asthma, it is important to understand its potential limitations when examining changes in airway responses and effects of novel therapies.

In this study, pulmonary gas trapping was examined as a potential index of airway hyperresponsiveness to methacholine in a murine model of asthma. First, we compared methacholine-induced excised lung gas volume changes in awake ovalbumin-sensitized, ovalbumin-exposed (Positive Control) and ovalbumin-sensitized, sodium chloride-exposed (Negative Control) mice. Second, we related increases in methacholine-induced pulmonary gas trapping with changes in enhanced pause in ovalbumin-sensitized and nonsensitized mice. To our knowledge, this is the first report examining excised lung gas volume and enhanced pause in the same animal. Third, we examined the ability of the inhaled glucocorticosteroid budesonide, which is used in the treatment of human asthma (Kamada et al., 1996; Barnes et al., 1998), to modify airway responses to methacholine and airway inflammation in this model. Finally, we compared the effect of the proliferator-activated receptor-γ (PPAR-γ) agonist ciglitazone to budesonide in light of recent studies suggesting ciglitazone showed efficacy in asthmatic mice (Woerly et al., 2003; Honda et al., 2004).

2. Methods

2.1. Animals

Inbred male BALB/c mice, 7–9 weeks of age, were purchased from Harlan (Indianapolis, IN). The animal room was maintained at 22–24 °C with a relative humidity of 35–70% and a daily light–dark cycle of 0600–1800 h light. Food (laboratory rodent diet 5001, PMI Feeds, St. Louis, MO) and water were supplied ad libitum. The weight range of mice was 21 to 33 g. Experimental protocols and procedures were approved by the Eli Lilly and Company Institutional Animal Care and Use Committee. The investigation fully conformed with the revised Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, *Research Guide for Care and Use of Laboratory Animals*.

2.2. Ovalbumin sensitization

Mice were immunized on Days 1 and 14 by intraperitoneal injection with 20 μg of ovalbumin (Sigma Chemical Company, St. Louis, MO) suspended in 0.1 ml Imject® Alum (Pierce Chemical Company, Rockford, IL). On Days 25–27, ovalbumin-sensitized mice were placed in a Plexiglas chamber (22.3 cm × 9.4 cm × 10.2 cm) and then exposed to an aerosol of ovalbumin (50.0 mg/ml in water) for 20 min daily. These mice were designated Positive Control. Other ovalbumin-sensitized mice were exposed to an aerosol of sodium chloride (50.0 mg/ml in water) in a separate Plexiglas chamber for 20 min daily on Days 25–27 and were designated Negative Control. The ovalbumin and sodium chloride aerosols were generated with a Pari ProNeb-Ultra nebulizer (PARI Respiratory Equipment, Inc., Monterey, CA). Airflow through the nebulizer was 11.4 L/min and usable nebulizer output was 0.2 ml/min.

2.3. Excised lung gas volume measurement

On Day 28, airway responses to methacholine chloride (Sigma Chemical Company, St Louis, MO) were assessed in Positive and Negative Control mice. Mice were placed in plastic restraining tubes (CH Technologies (USA) Inc., Westwood, NJ), which were attached via their nose-port to a 7.6-L polyvinyl chloride inhalation exposure chamber (Silbaugh et al., 1987). Methacholine aerosol solution concentrations, ranging from 0.3 to 30.0 mg/ml, were generated with a Lovelace nebulizer (Mercer et al., 1968). Chamber airflow was 10.0 L/min. Eight minutes after the start of the methacholine aerosol exposure, the mice were killed while breathing the chamber atmosphere with an intraperitoneal injection of 0.5 ml Fatal Plus (390 mg/ml sodium-pentobarbital, Vortech Pharmaceuticals, Dearborn, MI). After death, the abdomen was opened, the diaphragm was punctured and the lungs were allowed to deflate. The trachea of each mouse was cannulated with a 1.0 cm length of PE-90 tubing inserted to one-half its length. The lungs were then excised and carefully trimmed of nonpulmonary tissue. Excised lung gas volume, i.e., postmortem pulmonary gas trapping, an indicator of

in vivo airway obstruction, was measured by Archimedes' principle (Stengel and Silbaugh, 1986; Silbaugh et al., 1987) and was based on the stable amount of air trapped within the excised lungs at a transpulmonary pressure of 0.0 cm H_2O. The lungs were attached by the tracheal tube to a brass weight, which was then placed in a plastic cup, immersed in a beaker of saline on a stationary platform, and suspended from a hook at the top of a Mettler AE160 balance. By first taring the brass weight in saline, the lungs plus brass anchor gave a negative weight display in grams that closely approximates the ml of air trapped in the lungs. Because lung tissue density is similar to that for saline, the volume of air trapped in the lungs could then be determined. Excised lung gas volume measurements were completed within 30–45 min after death of the animal. A total of 39 mice (Positive Controls, $n=20$, and Negative Controls, $n=19$) were used to generate methacholine-induced pulmonary gas trapping concentration–response curves.

In additional groups of Positive Control and Negative Control mice, excised lung gas volume values following an 8-min methacholine (3.0 or 10.0 mg/ml) aerosol challenge were examined on Days 32 and 39, five and twelve days, respectively, after the last ovalbumin aerosol exposure. Excised lung gas volume measurements were made as described as above. A total of 134 mice (Positive Controls, $n=67$, and Negative Controls, $n=67$) were used to examine methacholine-induced pulmonary gas trapping on Days 28, 32, and 39.

2.4. Excised lung gas volume and enhanced pause

To examine the variability of the excised lung gas volume measurement, individual excised lung gas volume values of Positive and Negative Control mice were determined in nine studies performed over approximately 6 months. These mice were killed with an intraperitoneal injection of Fatal Plus 8.0 min after start of a 3.0 mg/ml methacholine aerosol exposure. Excised lung gas volume measurements were made as described above. A total of 100 mice (Positive Controls, $n=50$, and Negative Controls, $n=50$) were used to examine variability of methacholine-induced pulmonary gas trapping in nine studies over a 6-month period.

In other animals, to investigate the variability of the enhanced pause measurement, individual enhanced pause values of Positive and Negative Control mice were determined from nine studies performed over approximately 6 months. Positive and Negative Control mice were placed in a whole-body barometric plethysmograph (Buxco Electronics, Troy, NY) and exposed to a 30.0 mg/ml aerosol of methacholine for 2.0 min. The maximum enhanced pause value was obtained during the 3.5 min following the methacholine aerosol exposure. A total of 142 mice (Positive Controls, $n=72$, and Negative Controls, $n=70$) were used to examine the variability of the enhanced pause measurement following methacholine in nine studies over 6 months.

In a separate study, naïve mice were anesthetized with intraperitoneally administered urethane (1.25 g/kg) and a 30-gauge hypodermic needle containing heparinized saline (25 units/ml) was inserted into a tail vein. Animals were then placed in the whole-body barometric plethysmograph and baseline measurements of enhanced pause were made prior to the 4-min aerosol exposures of sodium chloride (300.0 mg/ml) or methacholine (30.0 or 300.0 mg/ml). Enhanced pause values were obtained immediately before each mouse was killed by 0.15 ml intravenous bolus of Fatal Plus. The lungs were then removed and excised lung gas volume measured as described above. A total of 14 naïve mice were used to directly compare methacholine-induced changes in enhanced pause and changes in excised lung gas volume values. The differences in methacholine solution concentrations used to examine methacholine-induced changes in excised lung gas volume and enhanced pause values reflect the dissimilarity of the two inhalation exposure systems.

2.5. Therapeutic intervention with budesonide and ciglitazone

Aerosols of budesonide (Sigma Chemical Company, St Louis, MO) and sodium chloride were generated with Lovelace nebulizers (Mercer et al., 1968). Chamber airflow was 10.0 L/min. Ovalbumin-sensitized mice were placed in plastic restraining tubes which were then attached via their nose-port to a 7.6-L polyvinyl chloride inhalation exposure chamber. The mice were treated daily for 4 days (Days 24–27) with an aerosol of budesonide (15.0 mg/ml) for 2 or 20 min to achieve a 10-fold separation in amount of inhaled budesonide between the two treatment groups. Other mice were exposed to a sodium chloride (15.0 mg/ml) aerosol for 20 min. The solvent for the budesonide and sodium chloride aerosol solutions was 40% dimethyl sulfoxide: 42% methanol: 18% water. One hour after treatment, ovalbumin-sensitized mice were exposed to an aerosol of ovalbumin (50.0 mg/ml) for 20 min on Days 25–27. Negative Control ovalbumin-sensitized mice were exposed to a sodium chloride aerosol (50.0 mg/ml) for 20 min. On Day 28, following an 8-min methacholine (3.0 mg/ml) aerosol challenge, mice were killed with a 0.5 ml intraperitoneal injection of Fatal Plus. Excised lung gas volumes were measured as described above. The lungs were then washed with 1.0 ml Dulbecco's phosphate buffered saline (without calcium chloride and magnesium chloride). The whole lung lavage fluid was centrifuged at 350 g for 10 min at 4 °C. The supernatant was removed and the pellet resuspended in 5.0 ml Dulbecco's phosphate buffered saline (without calcium chloride and magnesium chloride). A sample of the cells were stained with trypan blue to determine viability and the total cell count obtained by counting the cells with a hemocytometer. For differential cell counts, 0.2 ml aliquots of the resuspended cells were placed in a Shandon Cytospin (Shandon Southern Products Ltd., Astmoor Runcon Cheshire, UK) and centrifuged at 41 g for 10 min. Slides were stained with Wright–Giemsa and cells identified and counted by light microscopy. For each animal, the fraction of each cell type observed in differential counts was multiplied by the total cell count concentration and volume of whole lung lavage fluid recovered. In addition, spleens were removed and weighed. A total of 24 mice (Positive Controls, $n=18$ mice, and Negative Controls, $n=6$ mice) were used to examine the effect of inhaled budesonide on methacholine-induced pulmonary gas trapping,

whole lung lavage inflammatory cells, and spleen weights on Day 28.

In a similar study, ovalbumin-sensitized mice were treated daily for 4 days (Days 24–27) with aerosols of budesonide (10.0 mg/ml), ciglitazone (10.0 mg/ml) or sodium chloride (10.0 mg/ml) for 30 min. The solvent for the budesonide, ciglitazone and sodium chloride aerosol solutions was 40% dimethyl sulfoxide: 42% methanol: 18% water. On Days 25–27, 1 h after aerosol treatment, ovalbumin-sensitized mice were exposed to an aerosol of ovalbumin (50.0 mg/ml) for 20 min. Other ovalbumin-sensitized mice (Negative Control) were treated with sodium chloride (10.0 mg/ml in solvent) for 30 min. One hour later these mice were exposed to a sodium chloride aerosol (50.0 mg/ml) for 20 min. On Day 28, at the end of the 8-min methacholine (3.0 mg/ml) aerosol challenge, mice were killed with a 0.5 ml intraperitoneal injection of Fatal Plus. Excised lung gas volume values were determined, lungs washed and spleens weighed as described above. A total of 24 mice (Positive Controls, $n=6$, and Negative Controls, $n=6$) were used to examine the effect of inhaled budesonide ($n=6$) and ciglitazone ($n=6$) on methacholine-induced pulmonary gas trapping, whole lung lavage inflammatory cells, and spleen weights on Day 28.

In a separate study, ovalbumin-sensitized mice, placed in Plexiglas chambers, were treated daily for 3 days (Days 24–26) with aerosols of budesonide (5.0 mg/ml, $n=16$), ciglitazone (5.0×10^{-5} M, $n=16$) or sodium chloride (5.0 mg/ml, $n=16$) for 20 min immediately before and during the 20-min ovalbumin (50.0 mg/ml) aerosol exposure (Woerly et al., 2003; Honda et al., 2004). The solvent for the budesonide, ciglitazone and sodium chloride aerosol solutions was 40% dimethyl sulfoxide: 42% methanol: 18% water. Other ovalbumin-sensitized mice (Negative Control, $n=16$) were treated with sodium chloride (5.0 mg/ml in solvent) for 20 min and then immediately exposed to a sodium chloride aerosol (50.0 mg/ml) for 20 min. All aerosols were generated with a Pari ProNeb-Ultra nebulizer. On Day 27, methacholine (30.0 mg/ml) -induced changes in enhanced pause were examined. Like with methacholine, the differences in ciglitazone solution concentrations on methacholine-induced changes in excised lung gas volume and enhanced pause values reflect the dissimilarity of the two inhalation exposure systems.

2.6. Aerosol characterization

Aerosol particle size was determined by gravimetric analysis of cascade impactor samples (Mercer et al., 1970) of the chamber atmosphere. Mass mean aerodynamic diameters of ciglitazone and budesonide solutions were 4.7 and 5.1 μm, respectively, with geometric standard deviation values ranging from 2.0 to 2.6. The mass mean aerodynamic diameters of the sodium chloride aerosol was 1.5 μm, while the geometric standard deviation was 2.3. Total estimated inhaled doses of methacholine, budesonide, ciglitazone and sodium chloride were calculated based on nebulizer solution concentration, usable nebulizer output, inhalation time, chamber airflow, minute volume and animal body weight. The average minute volume value of 24.5 ml/min for the mouse was used (Guyton, 1947). The calculated respired doses of budesonide and ciglitazone (the dose of drug deposited on the trachea, bronchi and pulmonary regions) were determined from the regional deposition model of inhaled monodisperse aerosol particles in mice (Raabe et al., 1988). The total estimated inhaled doses and length of budesonide or ciglitazone treatment were similar to the intranasal doses and length of budesonide treatment described by Shen et al. (2002).

2.7. Statistical analyses

Results are presented as mean±standard error of the mean (S.E.M.) of 3 to 8 mice per group. Excised lung gas volume measurements are expressed in milliliters per kilogram (ml/kg) body weight. Enhanced pause values were averaged over 20-s intervals at selected times. Two-way repeated-measures analysis of variance was used to compare methacholine-induced changes in excised lung gas volumes and enhanced pause of Positive Control and Negative Control mice and changes in body weight of Positive Control, budesonide-treated, ciglitazone-treated and Negative Control mice. Bonferroni correction was performed to control for multiple comparisons. The curves were fitted using SAS software (SAS Institute, Cary, NC). Also, the relationship between log[excised lung gas volume] and log[enhanced pause] was examined using the Spearman rank order correlation. One-way analysis of variance was used to compare methacholine-induced changes in excised lung gas volume, whole lung lavage fluid inflammatory cells and spleen weights of Positive Control, budesonide-treated, ciglitazone-treated and Negative Control mice and Fisher's least significant difference test for all pairwise comparisons was performed when appropriate using SigmaStat for Windows (version 2.03; SPSS Science Inc., Chicago, IL). Comparisons were considered significant for P values of 0.05 or less. The relative inhibition of excised lung gas volume responses was determined by subtracting individual animal

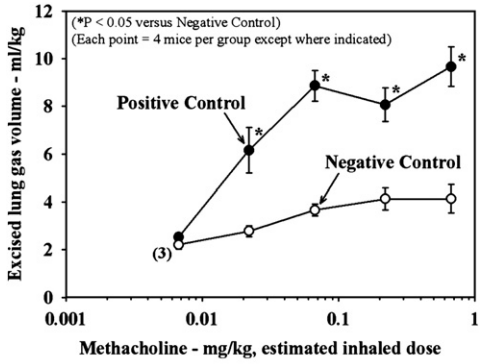

Fig. 1. Effect of methacholine aerosol on pulmonary gas trapping in Positive Control (ovalbumin-sensitized, ovalbumin-exposed) and Negative Control (ovalbumin-sensitized, sodium chloride-exposed) male BALB/c mice. Excised lung gas volume values are expressed in ml/kg. Each point represents the mean of 4 mice per group except where indicated and the vertical bars represent the S.E.M. for the number of animals. Absence of error bars indicates that the magnitude of error was less than the symbol size. Asterisks indicate significant difference between the excised lung gas volume values ($F=100.74$, $P<0.001$) for Positive and Negative Control mice.

Table 1
Relative ED_{200} values for methacholine producing changes in excised lung gas volume in Positive and Negative Control mice

Group	*n*	ED_{200} (95% confidence interval)
Positive Control	20	0.013 (0.0067–0.019) mg/kg
Negative Control	19	0.101 (0.033–0.340) mg/kg

ED_{200}, estimated inhaled dose of methacholine needed to increase excised lung gas volume to 200% of control; *n*, number of animals.

excised lung gas volume values from the average excised lung gas volume values of Positive Control mice and dividing by the difference between the means of Positive and Negative Control groups. Normalized values for excised lung gas volumes were multiplied by 100 to obtain percent inhibition or potentiation.

3. Results

3.1. Pulmonary gas trapping as an index of airway hyperresponsiveness to methacholine

Exaggerated abdominal breathing, i.e. dyspnea, was apparent in mice exposed to increasing aerosol solution concentrations of methacholine (0.3–30.0 mg/ml) 28 days after the first ovalbumin immunization. Methacholine bronchoprovocation produced a dose-dependent increase in pulmonary gas trapping in Positive ($n=20$) and Negative ($n=19$) Control mice (Fig. 1). At the time of excised lung gas volume measurements, the trachea and mainstem bronchi were not visibly obstructed. The pulmonary gas trapping produced by methacholine in Negative Control mice was similar to what we observed in naïve BALB/c mice from an earlier study (Stengel et al., 1995), suggesting that ovalbumin sensitization alone was insufficient to cause an increase in airway responsiveness to methacholine. After three daily ovalbumin aerosol exposures (Days 25–27), maximum excised lung gas volume values, of methacholine-exposed Positive Control mice were 1.3–2.4 times greater than those of Negative Control mice on Day 28. Also, Positive Controls were far more sensitive to methacholine than were Negative Controls when expressed as the percent of control excised lung gas volume values (1.94±0.24 ml/kg, $n=4$). The sensitivity to methacholine in Positive Controls was 7.8 times greater than that observed for the Negative Controls (Table 1). Thus, Positive Control mice showed both increased reactivity (greater

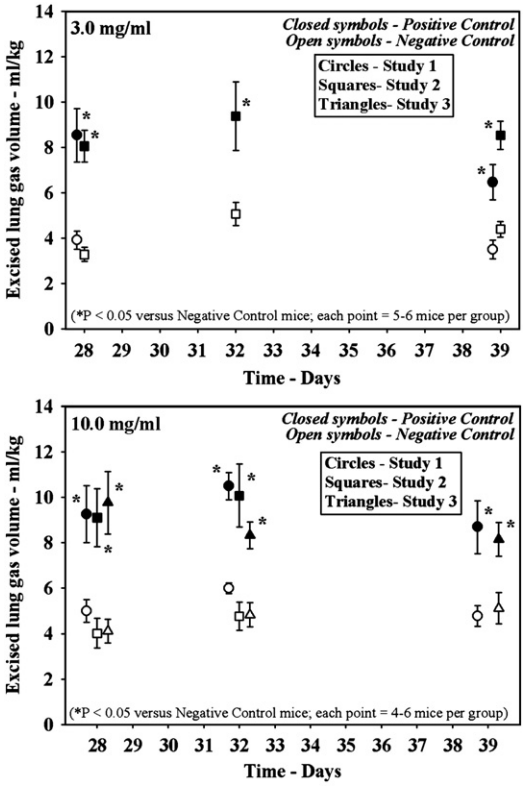

Fig. 2. Comparison of methacholine (3.0 mg/ml, upper panel)- and (10.0 mg/ml, lower panel) -induced postmortem pulmonary gas trapping between Positive and Negative Control mice 1, 5 or 12 days after the last ovalbumin or sodium chloride aerosol exposure. Each point corresponds to the mean of 4–6 mice per group and the vertical bars represent the S.E.M. for the number of animals. Asterisks indicate significant difference ($P<0.05$) between the excised lung gas volume values for Positive and Negative Control mice.

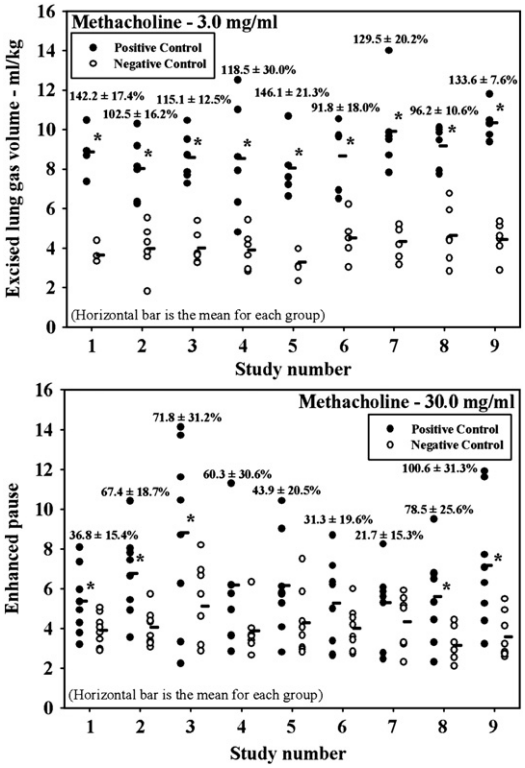

Fig. 3. Indirect comparison in pulmonary gas trapping (upper panel) and enhanced pause (lower panel) of Positive and Negative Control mice produced by methacholine. For each method, intra- and inter-study variability was assessed in nine experiments performed over approximately 6 months. Each point corresponds to an individual animal, and the horizontal bar represents the mean for each group. One hundred mice (Positive Controls, $n=50$ mice, and Negative Controls, $n=50$ mice) were used to examine methacholine-induced pulmonary gas trapping, while 142 mice (Positive Controls, $n=72$ mice, and Negative Controls, $n=70$ mice) were used to examine enhanced pause following methacholine. The percentages listed in each figure represent the relative increase in excised lung gas volume and enhanced pause values of Positive Control mice compared to Negative Control mice. Asterisks indicate significant difference ($P<0.05$) between the mean excised lung gas volume or mean enhanced pause values for Positive and Negative Control mice.

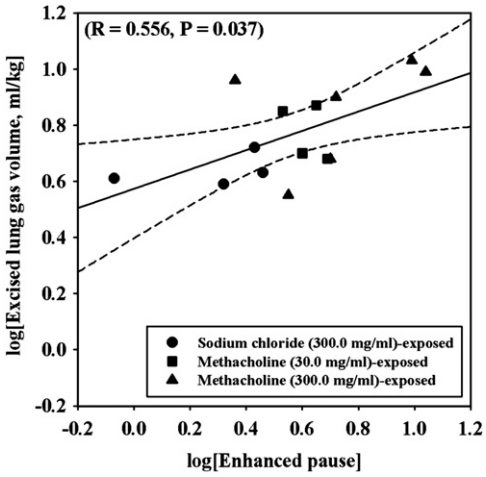

Fig. 4. Relationship of log[excised lung gas volume] with log[enhanced pause] obtained immediately prior to death in naïve mice ($n=14$). Pulmonary gas trapping was directly related to enhanced pause. Each point represents one animal: circle—sodium chloride (300 mg/ml); square—methacholine (30 mg/ml); and triangle—methacholine (300 mg/ml). The dashed lines represent the 95% confidence interval around the regression line.

maximum effect) and increased sensitivity (lower ED_{200} excised lung gas volume) to methacholine.

Next we determined if the increased airway responsiveness seen one day after exposure of three ovalbumin aerosols remained for an extended period of time. Positive ($n=67$) and Negative ($n=67$) Control mice were challenged with aerosols of methacholine (3.0 or 10.0 mg/ml) on Days 32 and 39. Fig. 2 illustrates that this increased airway responsiveness to aerosol solution concentrations of methacholine in Positive Control mice persisted out to Days 32 and 39, five and twelve days after the last of the three daily ovalbumin aerosol exposures. Results from one to three studies revealed consistency and reproducibility of the excised lung gas volume measurement as an index of increased airway responsiveness to methacholine (3.0 or 10.0 mg/ml) for groups of Positive Control mice challenged on Days 28, 32 or 39. From Days 32 and 39, excised lung gas volume values of methacholine-exposed Positive Control mice were 1.6–2.5 times greater than those of methacholine-exposed Negative Control mice (see Fig. 2). Furthermore in a separate experiment, the exaggerated airway response to inhaled methacholine (3.0 mg/ml) for Positive Control mice was found to occur as early as 24 h after only a single ovalbumin aerosol exposure. Under these circumstances, a 1.6-fold greater responsiveness was observed compared to Negative Control mice (data not shown).

3.2. Comparison of excised lung gas volume and enhanced pause values

Examining mean excised lung gas volume of Positive ($n=50$) and Negative ($n=50$) Control mice from nine studies conducted over 6 months showed methacholine-induced postmortem pulmonary gas trapping to be remarkably stable from study to study over this time period (Fig. 3, upper panel). Further, in the nine studies there was only one Positive Control mouse that overlapped with Negative Control mice for individual excised lung gas volume values. This was observed comparing data from 116 ovalbumin-sensitized mice. In contrast, mean enhanced pause values of methacholine-challenged Positive ($n=72$) and Negative ($n=70$) Control mice showed tremendous variation in studies conducted over a 6 month period. Unlike the excised lung gas volume values, there was a substantial overlap of the individual enhanced pause values of Positive and Negative Control mice (Fig. 3, lower panel). Because of this overlap in enhanced pause data only five of nine studies showed significant difference between Positive and Negative Control Groups.

We used urethane-anesthetized naïve mice ($n=14$) to compare methacholine-induced changes in airway responses measured by enhanced pause and excised lung gas volume in the same animals. Mice were placed in the whole-body barometric plethysmograph and after baseline enhanced pause measurements were obtained, challenged with sodium chloride (300.0 mg/ml) or methacholine (30.0 or 300.0 mg/ml) aerosols for 4 min and then immediately killed with Fatal Plus and the lungs were removed to measure excised lung gas volume. The last enhanced pause value prior to the Fatal Plus injection was used to compare with the excised lung gas volume measurement. Baseline enhanced pause values of 1.76 ± 0.24, 1.51 ± 0.15 and 1.39 ± 0.20, were obtained for sodium chloride (300.0 mg/ml), methacholine (30.0 mg/ml) and methacholine (300.0 mg/ml) challenges, respectively, and these were not significantly different from each other ($F=0.797$, $P=0.475$). In urethane-anesthetized naïve mice, postmortem pulmonary gas trapping correlated with enhanced pause measurements (Fig. 4). However, approximately half of the values fell outside the 95% confidence interval following methacholine challenge.

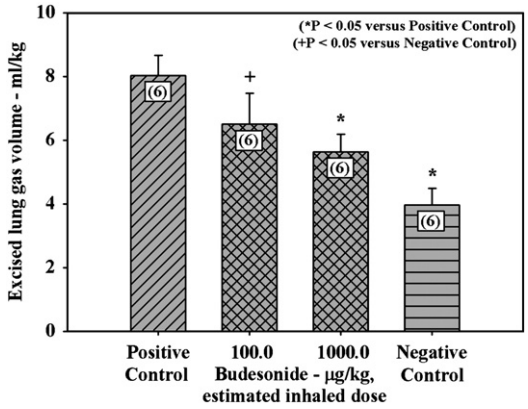

Fig. 5. The effect of daily treatment for 4 days with aerosols of budesonide (100.0 or 1000.0 µg/kg) or solvent on ovalbumin-induced airway responsiveness to methacholine, 24 h after ovalbumin aerosol exposure. Negative Control mice (horizontal striped bar) were exposed to the solvent aerosol daily for 4 days and then killed 24 h later following an 8-min methacholine aerosol exposure. Each bar represents the mean±S.E.M. of 6 mice per group. One-way analysis of variance was used to compare excised lung gas volume values among Positive Control, budesonide-treated and Negative Control mice ($F=5.875$, $P=0.005$) and Fisher's least significant difference for all pairwise comparisons was performed when appropriate. Asterisk indicates significant difference versus Positive Control mice and the cross denotes significant difference versus Negative Control mice.

3.3. Effect of inhaled budesonide on body weight, airway responsiveness to methacholine, spleen weight and inflammatory cells

Following four days of aerosol exposures, body weights of budesonide-treated mice ($n=12$) did not differ ($F=0.53$, $P=0.67$) from those of either Positive ($n=6$) or Negative ($n=6$) Control mice (data not shown). Inhaled budesonide (100.0 and 1000.0 μg/kg, total estimated inhaled dose) dose-dependently reduced methacholine-induced changes in excised lung gas volume values (Fig. 5). At the highest dose, budesonide (1000.0 μg/kg, total estimated inhaled dose) the pulmonary gas trapping response to methacholine was significantly less than that of Positive Control group, and was not different from excised lung gas volume values of the Negative Control mice. The percent reduction in airway responsiveness to methacholine produced by this dose of budesonide was 59.0±13.9% and the lower dose of budesonide produced a 37.5±24.1% reduction which was not statistically significant. Inhaled budesonide dose-dependently reduced spleen weights of budesonide-treated mice (data not shown), indicating the occurrence of systemic exposure from the aerosol delivery. In addition, inhaled budesonide caused a dose-dependent reduction in both whole lung lavage fluid eosinophils and neutrophils (Fig. 6). Both the 100.0 and 1000.0 μg/kg estimated inhaled doses of budesonide significantly inhibited whole lung lavage neutrophil infiltration, while only the 1000.0 μg/kg estimated inhaled dose of budesonide significantly prevented whole lung lavage eosinophil infiltration ($P<0.05$). These results taken together indicate that attenuation of airway hyperresponsiveness

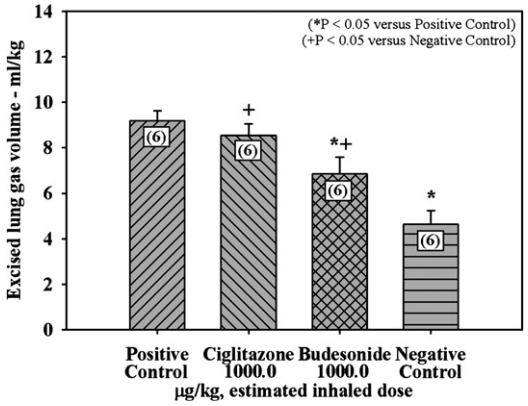

Fig. 7. The effect of daily treatment for 4 days with aerosols of budesonide (1000 μg/kg), ciglitazone (1000 μg/kg) or solvent on methacholine-induced changes in excised lung gas volumes, 24 h after ovalbumin aerosol exposure. Negative Control mice (horizontal striped bar) were exposed to the solvent aerosol daily for 4 days and then killed 24 h later following an 8-min methacholine aerosol exposure. Each bar represents the mean±S.E.M. of 6 mice per group. One-way analysis of variance was used to compare excised lung gas volume ($F=12.232$, $P<0.001$) values among Positive Control, budesonide-treated and Negative Control mice and Fisher's least significant difference for all pairwise comparisons was performed when appropriate. Asterisk indicates significant difference versus Positive Control mice and the cross denotes significant difference versus Negative Control mice.

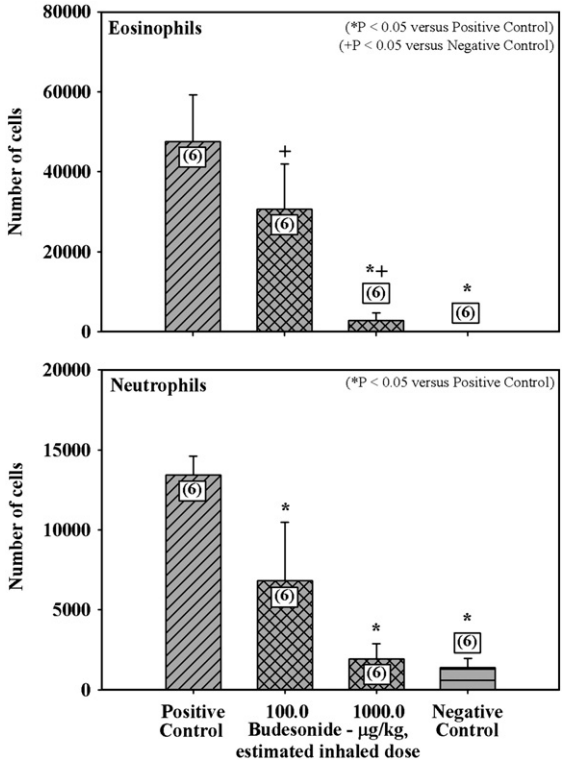

Fig. 6. The effect of daily treatment for 4 days with aerosols of budesonide (100.0 or 1000.0 μg/kg) or solvent on eosinophils (upper panel) and neutrophils (lower panel) of ovalbumin-sensitized mice. Each bar represents the mean±S.E.M. of 6 mice per group. One-way analysis of variance was used to compare eosinophils ($F=9.914$, $P<0.001$) and neutrophils ($F=7.780$, $P=0.001$) among Positive Control, budesonide-treated and Negative Control mice and Fisher's least significant difference for all pairwise comparisons was performed when appropriate. Asterisk indicates significant difference versus Positive Control mice and the cross denotes significant difference versus Negative Control mice.

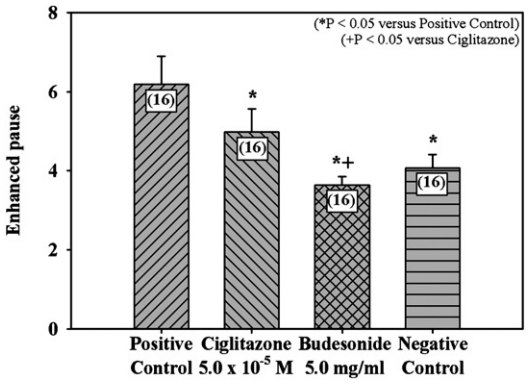

Fig. 8. The effect of daily treatment for 3 days with aerosols of budesonide (5.0 mg/ml), ciglitazone (5.0×10^{-5} M) or solvent on methacholine (30.0 mg/ml) -induced changes in enhanced pause, 24 h after ovalbumin aerosol exposure. Negative Control mice (horizontal striped bar) were exposed to the solvent aerosol daily for 3 days and then challenged with methacholine (30.0 mg/ml) 24 h after sodium chloride aerosol exposure. Each bar represents the mean±S.E.M. of 16 mice per group. One-way analysis of variance was used to enhanced pause ($F=8.62$, $P<0.001$) values among Positive Control, budesonide-treated and Negative Control mice and Fisher's least significant difference for all pairwise comparisons was performed when appropriate. Asterisk indicates significant difference versus Positive Control mice and the cross denotes significant difference versus Negative Control mice.

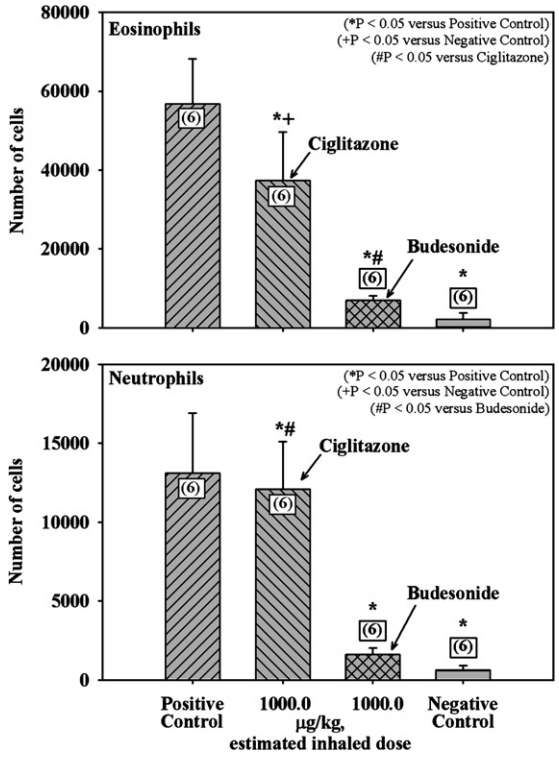

Fig. 9. The effect of daily treatment for 4 days with aerosols of budesonide (1000 µg/kg), ciglitazone (1000 µg/kg) or solvent on eosinophils (upper panel) and neutrophils (lower panel) of ovalbumin-sensitized mice. Each bar represents the mean±S.E.M. of 6 mice per group. One-way analysis of variance was used to compare eosinophils (F=21.456, P<0.001) and neutrophils (F=6.244, P=0.004) among Positive Control, budesonide-treated, ciglitazone-treated and Negative Control mice and Fisher's least significant difference for all pairwise comparisons was performed when appropriate. Asterisk indicates significant difference versus Positive Control mice, the cross denotes significant difference versus Negative Control mice and the pound denotes significant difference versus budesonide (1000 µg/kg) -treated mice.

to methacholine is associated with suppression of allergen-induced airway inflammation.

3.4. Effect of inhaled budesonide and ciglitazone on body weight, airway responsiveness to methacholine, spleen weight and inflammatory cells

In a separate study we compared the effects of aerosolized budesonide (1000.0 µg/kg, total estimated inhaled dose, n=6) with aerosolized ciglitazone (1000.0 µg/kg, total estimated inhaled dose, n=6) administered for four days. Body weights of budesonide- and ciglitazone-treated mice were not different on any of the four days of aerosol exposures from either Positive (n=6) or Negative Control (n=6) mice (data not shown). Budesonide (1000.0 µg/kg, total estimated inhaled dose) produced a similar significant reduction (51.0±16.1%) in methacholine-induced pulmonary gas trapping as previously observed whereas ciglitazone (1000.0 µg/kg, total estimated inhaled dose) was without effect (13.9±11.2%) as illustrated in Fig. 7. The decrease in spleen weights was an effect observed following inhalation of budesonide, but not ciglitazone (data not shown). Since aerosolized ciglitazone was shown to decrease methacholine-induced changes in enhanced pause (Woerly et al., 2003; Honda et al., 2004), but failed to modify methacholine-mediated excised lung gas volumes (this study), we examined the effect of ciglitazone (5.0×10^{-5} M, n=16) and budesonide (5.0 mg/ml, n=16) on changes in enhanced pause following a methacholine (30.0 mg/ml) aerosol challenge in ovalbumin-sensitized, ovalbumin-exposed mice. Under conditions similar to those of Woerly et al. (2003) and Honda et al. (2004), both ciglitazone and budesonide reduced methacholine-related increases in enhanced pause (Fig. 8). As illustrated in Fig. 9, budesonide markedly reduced both whole lung lavage eosinophil and neutrophil infiltration by 87.1±1.9 and 87.5±3.2%, respectively. In contrast, ciglitazone modestly reduced whole lung lavage fluid eosinophil infiltration by 34.3±21.8%, but not whole lung lavage neutrophil infiltration (7.7±23.0%).

4. Discussion

This study clearly demonstrates that pulmonary gas trapping is a sensitive indicator of in vivo changes in airway responsiveness to methacholine in a murine asthma model. Following methacholine bronchoprovocation, excised lung gas volume increases were likely caused by increases in the amount of gas trapped distal to occluded airways, similar to air trapping and higher residual volumes observed during acute episodes of human asthma (Woolcock and Read, 1985). Excised lung gas volume measurements were made soon after inhalation of the rapidly acting bronchoconstrictive agonist methacholine, strongly supporting airway smooth muscle constriction as the primary cause of pulmonary gas trapping. However, we cannot discount the possibility that increased airway secretions and/or interstitial edema also may have contributed to the airway obstruction. Since trachea and mainstem bronchi were visibly patent during excised lung gas volume measurements, sites of airway closure had to occur distal to these airways.

Using excised lung gas volume, we demonstrated airway hyperresponsiveness to methacholine was apparent following one ovalbumin exposure in ovalbumin-sensitized mice. Following three consecutive ovalbumin exposures, airway hyperresponsiveness to methacholine was evident the following day (Day 28) and was maintained for five (Day 32) and twelve (Day 39) days without re-exposure to ovalbumin, indicating airway hyperresponsiveness to methacholine is more than an acute phenomenon. However, additional work needs to be done to examine the usefulness of this approach as a chronic model of asthma.

This study demonstrated that the excised lung gas volume technique is very reproducible compared to enhanced pause. There was only one incidence of overlap in individual excised lung gas volume values of Positive and Negative Controls in nine studies. All excised lung gas volume studies showed statistical separation between Positive and Negative Controls. In contrast, there was marked overlap of individual enhanced pause values of Positive and Negative Controls in all nine studies, with only five studies showing statistical separation. It is unlikely differences in ovalbumin-sensitization and

-exposures would account for this variability observed in Positive Controls, since ovalbumin-sensitization and -exposures were identical in both excised lung gas volume and enhanced pause studies.

The excised lung gas volume technique, like more traditional methods for measuring airway function, is unable to determine the exact mechanism or location of airway obstruction. Also, there are advantages and limitations of the excised lung gas volume measurement. It is a rapid, quantitative and sensitive technique requiring minimal surgical or anesthetic preparation, which could confound experimental results. Also, since several treatment groups can be exposed simultaneously, the effects of temporal variations in animal response and potential run-to-run differences in aerosol properties can be minimized. The major limitation of the excised lung gas volume technique is that this single-time-point measurement requires the death of the animal.

Previously, we reported dose-related excised lung gas volume increases after methacholine challenge in naïve male A/J, BALB/c and C3H/HeJ mice (Stengel et al., 1995; Yiamouyiannis et al., 1995). Other investigators have shown greater excised lung gas volume increases of ovalbumin-sensitized, ovalbumin-exposed C57/BL6 mice following aerosolized methacholine compared to Negative Control littermates (Hatfield et al., 1997; Yiamouyiannis et al., 1999; Schramm et al., 2000). However, only one (Hatfield et al., 1997) or two (Yiamouyiannis et al., 1999; Schramm et al., 2000) methacholine aerosol concentrations were examined. Further, there was no indication of the reproducibility of the excised lung gas volume measurement or demonstration of the maximal airway obstructive response to methacholine in Positive and Negative Control mice.

Similar to our previous investigations examining antagonism of methacholine-induced airway obstruction in guinea pigs (Silbaugh et al., 1987; Stengel et al., 1993), we used methacholine-induced pulmonary gas trapping in ovalbumin-sensitized mice to study pharmacological intervention of therapeutic agents. Thus, we evaluated the effect of inhaled budesonide in ovalbumin-sensitized mice and found budesonide dose-dependently reduced airway hyperresponsiveness to methacholine and whole lung lavage eosinophils and neutrophils. Estimating a total inhaled dose of 1000.0 μg/kg for budesonide, and assuming 2% pulmonary deposition for a 5.0 μm particle in mice (Raabe et al., 1988), the calculated respired dose would be 20.0 μg/kg, which is approximately 10-fold higher than the dose used to treat mild-to-moderate asthmatics. The inhalation route was chosen since in asthmatics inhaled glucocorticosteroids have been found to be especially effective in reducing both allergen-induced airway hyperresponsiveness to methacholine and airway eosinophilia (Gauvreau et al., 1996; van Rensen et al., 1999; Kelly et al., 2000).

However, potential systemic events are a major concern of inhaled glucocorticosteroids (Kamada et al., 1996). One possible adverse effect due to systemic exposure following inhaled glucocorticosteroids is adrenal suppression. For instance, inhaled doses of budesonide greater than 800 μg/d have been shown to reduce plasma cortisol in both normal male volunteers (Boorsma et al., 1996; Donnelly et al., 1997) and stable asthmatics (Clark and Lipworth, 1997).

Our observation that inhaled budesonide significantly decreased spleen weights indicated systemic exposure to budesonide. Similar spleen weight reductions following aerosolized budesonide were observed by other investigators (Wattenberg et al., 1997, 2000) who found inhaled budesonide (10.0 to 126.0 μg/kg) reduced spleen weights of female A/J mice, doses which also reduced pulmonary adenoma tumor formation. Thus, the dose-dependent effect we observed with inhaled budesonide on methacholine-induced excised lung gas volume changes and inflammatory cell recruitment was likely due in part to its systemic action.

The lack of efficacy with the PPAR-γ agonist ciglitazone on methacholine-induced excised lung gas volume was surprising since inhaled ciglitazone reduced allergen-induced airway hyperresponsiveness to methacholine in ovalbumin-sensitized mice using enhanced pause (Woerly et al., 2003; Honda et al., 2004; this study). Since enhanced pause was first introduced (Hammelmann et al., 1997), it has undergone tremendous scrutiny and concerns have been raised regarding its validity as an index of airway mechanics compared to other methods for assessing pulmonary function such as pulmonary gas trapping (Drazen et al., 1999). Further, recent investigations indicate enhanced pause is more likely related to changes in ventilatory timing rather than changes in airway resistance (Mitzner and Tankersley, 1998; Lundblad et al., 2002). Thus, if the inhibitory effect of inhaled ciglitazone (Woerly et al., 2003; Honda et al., 2004) was a consequence of changes in ventilation instead of changes in airway mechanics, alterations in ventilatory parameters would not have been detected by measuring excised lung gas volumes.

Alternatively, differences in aerosol exposure conditions could also account for the apparent lack of effect of inhaled ciglitazone on methacholine-induced excised lung gas volume changes versus the effectiveness of inhaled ciglitazone on methacholine-mediated enhanced pause changes (Woerly et al., 2003; Honda et al., 2004; this study). Aerosol exposure conditions among studies do not provide sufficient detail to calculate an estimated inhaled dose of ciglitazone.

Also, it is possible that the pulmonary actions of aerosolized ciglitazone were short-acting, although we are unaware of information indicating ciglitazone is short-acting. We waited 1 h after the 30-min ciglitazone aerosol to begin ovalbumin exposure, whereas Woerly et al. (2003) and Honda et al. (2004) aerosolized ciglitazone for 20 min immediately before and during the 20-min ovalbumin exposure, suggesting that coadministration of ciglitazone with ovalbumin might optimize its effectiveness. However, the decreased eosinophilic infiltration we observed in ciglitazone-treated mice was similar to that reported by Woerly et al. (2003) indicating that although aerosol exposures differed considerably, ciglitazone was still capable of modifying airway eosinophilia.

Although we found that ciglitazone reduced eosinophilia, it had no effect on neutrophil recruitment. This is supported by a recent study examining the effect of another PPAR-γ agonist, GI 262570, on airway inflammation in a murine model of asthma (Trifilieff et al., 2003). These investigators showed that the intranasally administered PPAR-γ agonist inhibited

eosinophilia, but not neutrophilia. This raises the possibility that neutrophilic inflammation may play a role in the airway hyperresponsiveness to methacholine in our Positive Control mice and could explain the effectiveness of budesonide at the high inhaled doses used in the current study. With regard to this, recent reports examining either biopsy or sputum samples from severe asthmatics suggest the involvement of neutrophilic inflammation in persistent asthma (Jatakanon et al., 1999; Wenzel et al., 1999; Gibson et al., 2001; Green et al., 2002). However, asthmatic patients with neutrophilic airway inflammation tend to respond poorly to inhaled glucocorticosteroids (Green et al., 2002).

Recently, Taube et al. (2004) concluded that neutrophils did not alter the progression of airway hyperresponsiveness to methacholine in their ovalbumin-sensitized mice based on the lack of inhibition of early airway neutrophilic inflammation with either interleukin-1 receptor antagonist or anti-interleukin-18 antibody. Differences in ovalbumin challenge could account for this apparent discrepancy between their study and ours since they used only a single intranasal administration of ovalbumin, while we exposed sensitized mice to ovalbumin aerosols for three consecutive days. Another possible explanation is the time point when whole lung lavage neutrophils were examined following ovalbumin challenge. Taube et al. (2004) measured neutrophilia 48 h after the single intranasal administration of ovalbumin, whereas we determined neutrophilia 24 h following the last ovalbumin aerosol exposure.

In conclusion, we observed that excised lung gas volume measurements can differentiate airway responses to methacholine of ovalbumin-sensitized, ovalbumin-exposed and ovalbumin-sensitized, sodium chloride-exposed mice. Second, determining pulmonary gas trapping in mice is a simple, rapid and reliable way of measuring airway obstruction in this species especially when compared to enhanced pause. Third, inhaled budesonide dose-dependently reduced allergen-induced airway hyperresponsiveness to methacholine and airway eosinophilia, effects consistent with its therapeutic action in the treatment of human asthma. Finally, this study highlights the importance of running a positive comparator such as budesonide when examining a novel treatment like ciglitazone.

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