

Allergen challenge during halothane compared to isoflurane anesthesia induces a more potent peripheral lung response

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ABSTRACT

Allergen instillation in anaesthetized vs. awake animals results in increased distribution of allergen in the lung. Halothane is a more potent bronchodilator of the small airways than isoflurane. As small airways contribute to asthma pathogenesis, we questioned whether intranasal challenge under halothane vs. isoflurane anesthesia would lead to an increase in allergen deposition in the lung periphery and, consequently, an enhanced allergic response. C57Bl/6 mice were sensitized twice and repeatedly challenged with ovalbumin (OA) under halothane or isoflurane anesthesia. After OA-challenge, *in vivo* lung function was measured and BAL performed. Peribronchial and peripheral inflammation, cytokine mRNA production and collagen deposition were assessed. Airway hyperresponsiveness, BAL eosinophilia, peripheral lung inflammation, IL-5 mRNA production and collagen deposition were significantly increased in halothane OA-challenged compared to isoflurane OA-challenged mice. Airway challenge induced a higher level of airway hyperresponsiveness, inflammation and remodeling under halothane than isoflurane anesthesia in a murine model of asthma. These differences may be due to increased allergen deposition in the small airways.

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1. Introduction

Animal models are commonly used for studies of the pathogenesis of asthma and for the development of novel therapies (Bates et al., 2009). Different routes of allergen administration have been used in order to induce an asthma-like phenotype in experimental animals, with features such as airway inflammation, eosinophilia, IgE and Th2 cytokine production, airway remodeling and hyperresponsiveness. Among the various modalities of challenge, the intranasal route is an effective, noninvasive, convenient and therefore, frequently used technique to deliver allergen to the airways and induce animal models of asthma (McCusker et al., 2002; Pinelli et al., 2009; Southam et al., 2002; Zhang et al., 1997).

Depending on the experimental conditions, allergen can be predominantly deposited in the upper airways and/or lung periphery. Previous studies have evaluated allergen distribution following intranasal challenges with various solution volumes, in awake

animals and in anesthetized animals under different anesthetic drugs (Eyles et al., 1999; Soto-Montenegro et al., 2009; Southam et al., 2002; Tomaki et al., 2000). During intranasal challenge, instillation volumes and challenges performed under anesthetized vs. awake conditions influenced the relative solution distribution, i.e., between the upper respiratory tract and lower respiratory tract and gastrointestinal tract, whereas body position did not (Soto-Montenegro et al., 2009; Southam et al., 2002). Furthermore, when intranasal challenge was performed under three different anesthetic drugs, tribromoethanol, isoflurane and halothane, the relative solution distribution to the lungs was not influenced by type of anesthesia (Southam et al., 2002). Conversely, when mice were challenged with a solution containing bacteria under different anesthetic drugs, inhaled isoflurane and parenteral ketamine/xylazine, pulmonary deposition was greater with inhaled anesthesia (Miller et al., 2012). Moreover, even using the same challenge protocol, intranasal challenges induced distinct patterns of airway remodeling in different sized airways (Hirota et al., 2006). Although intranasal challenges have been widely used, there are few data on potential differences in allergen deposition in the airways and its effect on asthma-like characteristics, depending upon the anesthetic drug used in these animal models.

Several studies suggest that the small airways contribute to asthma pathogenesis (Despas et al., 1972; Gelfand and Kraft, 2009; Hyde et al., 2009). Considering that studies of small airway involvement in clinical asthma are limited due to the difficulty in obtaining

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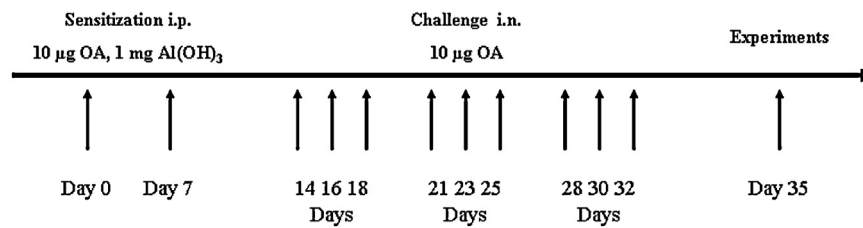


Fig. 1. Allergic sensitization and challenge protocol. Mice were sensitized with ovalbumin (OA) and adjuvant aluminum hydroxide on two separate days, one week apart. One week after the second sensitization, mice were challenged via the intranasal route with OA three times per week for three weeks under light isoflurane and halothane anesthesia. Seventy-two hours after the final challenge, experiments were performed. The control groups were sensitized and challenged with sterile saline solution in the same days.

adequate tissue from the lung periphery (Hyde et al., 2009), animal models are thus an important tool to evaluate the influence of small airways in asthma pathophysiology and treatment. To our knowledge, there is no specific animal model to evaluate the contribution of small airways in asthma. While isoflurane and halothane both have bronchodilator actions, halothane is a more potent bronchodilator of the small airways (Brown et al., 1993; Mercier et al., 2002). Therefore, we hypothesized that intranasal challenges during halothane anesthesia could result in enhanced allergen deposition in the lung periphery and induce a more potent peripheral airway response and potentially provide a reasonable animal model for study of the peripheral airway response in an animal model of asthma.

2. Methods

2.1. Mice

C57Bl/6 mice, 6–8 weeks, housed in a regular animal facility at McGill University (Montreal, QC, Canada), were studied. Protocols and procedures were evaluated and accepted by the animal ethics committee of McGill University.

2.2. Allergic sensitization and challenge protocol

Allergic sensitization and challenge protocol are shown in Fig. 1. Mice were sensitized with an intra-peritoneal (i.p.) injection containing 10 µg ovalbumin (OA) (Sigma, St Louis, MO, USA) and 1 mg adjuvant aluminum hydroxide, Al(OH)₃ (Fisher Scientific, Ottawa, QC, Canada) on two separate days, one week apart. One week after the second sensitization, mice were challenged via the intranasal route with instillation of 50 µl of OA (10 µg) three times per week for three weeks under isoflurane or halothane anesthesia. For isoflurane protocol, a mixture of isoflurane (3–5%) and oxygen was delivered by a vaporizer in a closed box until mice become lightly anesthetized. Anesthesia with halothane was performed in a box until a similar level of anesthesia was achieved. Seventy-two hours after the final challenge, experiments were performed. The control groups were sensitized and challenged with sterile saline (Sal) solution on the same days.

2.3. Measurement of in vivo respiratory parameters – Forced Oscillation Measurements

Measurements of respiratory parameters were performed as previously described (Marchica et al., 2011; Pinelli et al., 2009). Briefly, mice were anesthetized i.p. with an injection of xylazine (10 mg/kg) and pentobarbital (30 mg/kg). After 5 min, they were tracheostomized and an 18-gauge metal cannula was inserted into the trachea and firmly tied. Mice were connected via the tracheal cannula to a computer controlled small animal ventilator (FlexiVent, Scireq, Montreal, QC, Canada) and ventilated at 150

breaths/minute and a PEEP of 3 cm H₂O. Animals were paralyzed with an injection i.p. of pancuronium bromide (1.2 mg/kg), prior to the initiation of mechanical measurements. Data were obtained at baseline and after delivery of increasing concentrations of methacholine (Sigma, St Louis, MO, USA) aerosol (6.25, 12.5, 25 and 50 mg/ml) for 10 seconds via an ultrasonic nebulizer (Hudson RCI, Teleflex Medical, Temecula, CA, USA). The peak response of each parameter was determined and used for analyses (coefficient of determination (C.O.D.) greater than 0.8).

Respiratory mechanics were measured using the constant phase model, a mathematical model of the lung developed by Hantos et al. (1992). This model allows partitioning of changes into large airway and peripheral compartments, by measuring central airway resistance (R_n), tissue resistance (G) and tissue elastance (H), and provides valuable information concerning which lung components contribute to airway hyperresponsiveness (Hantos et al., 1992).

During the forced oscillation technique, the computer-controlled piston applies 13 sinusoidal components with mutually prime frequencies ranging from 1 to 20.5 Hz, as an input signal during a period of 3 s (quick prime-3 perturbation). Multiple linear regression is used to fit data to the constant phase model of the lung using the following equation:

$$Z_{RS}(f) = R_{aw} + j2\pi f l + \frac{G_{ti} - jH_{ti}}{(2\pi f)^\alpha},$$

where Z_{RS} is respiratory system impedance, R_{aw} is airway resistance, j is the positive square root of -1 , $j2\pi f l$ represents the imaginary part, l is airway inertance, G_{ti} (tissue damping) reflects energy dissipation and, in a homogeneous lung, is closely related to peripheral airway and tissue resistance, H_{ti} reflects energy conservation in the lung tissues and is closely related to tissue elastance, $\alpha = (2/\pi)\tan^{-1}(H/G)$, and f is respiratory frequency (Hantos et al., 1992). Tissue hysteresivity (η) was calculated as G/H (Fredberg and Stamenovic, 1989).

2.4. Bronchoalveolar lavage (BAL) fluid

Following lung mechanics, BAL was carried out twice by instilling and subsequently withdrawing 1 ml of cold saline via the tracheal cannula. BAL cytospin slides were prepared and then fixed and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI, USA). Differential BAL cell counts were determined (sampling a total of three hundred cells).

2.5. Tissue preparation and fixation

Following the in vivo measurements, the chest wall was opened and lungs were excised. The right lobe was stored in RNAlater (Qiagen, Mississauga, ON, Canada) for mRNA analysis. The left lung was inflated with 10% phosphate-buffered formalin at a transpulmonary pressure of 25 cmH₂O for 25 min. The tissue was processed and subsequently embedded in paraffin blocks.

Table 1
Primer sequence.

Primer name	Sequence (5'–3')
IL-4	Forward – CATCTGCTCTTCTTCTCG Reverse – TCTGTGGTGTCTTCGTTGC
IL-5	Forward – AGGCTTCTGTCCCTACTCA Reverse – TCCTCGCCACACTTCTCTTT
IL-13	Forward – TGAGCAACATCACACAAGACC Reverse – TACAGAGGCCATGCAATATCC
S9	Forward – AAGCAACTGATTGAACCCGTGCAG Reverse – ATCTTCCCCTTCCTGCTCATAA

2.6. Histopathology

Sagittal tissue sections with a thickness of 5 μm were cut, and hematoxylin and eosin (H&E) and picosirius staining were performed. Morphometric analyses were performed on airways that had intact epithelial lining as well as a minimum to maximum internal diameter (D2/D1) greater than or equal to 0.5. Generally, 4–5 airways were examined per mouse. An Olympus light microscope (Olympus, ON, Canada) was used and images were captured with the CoolSnap PROc color camera (Media Cybernetics, Baltimore, MD, USA). Measurements were obtained using the Image pro-PLUS software (Version 5.2, Media cybernetics L.P., Silver Spring, MD, USA).

In order to quantify peribronchial inflammation, the number of inflammatory cells, including granulocytes and mononuclear cells, was counted in an area circumscribed from the basement membrane to the alveolar attachments, as previously described (Marchica et al., 2011; Pinelli et al., 2009). To further evaluate the involvement of lung periphery, the point counting method was used. A grid of 121 (11 $\times$ 11) points was superimposed onto each captured image and the number of reference points that fell on inflammatory cells was counted and expressed as the number of inflammatory cell points/total points.

Analysis of positive staining for collagen (picosirius staining) was limited to the airway area. Using the Image Pro-Plus software, the color of the positive stain was selected, and the positive stained area and airway area were measured. Results were expressed as the area of positive stain/airway area.

The mean airway size used for determination of inflammation in isoflurane and halothane OA-challenged mice, were $413 \pm 14 \mu\text{m}$ and $432 \pm 12 \mu\text{m}$, respectively ($p > 0.05$), and for collagen staining were $626 \pm 39 \mu\text{m}$ and $778 \pm 46 \mu\text{m}$, respectively ($p > 0.05$).

2.7. RNA extraction and quantitative real-time PCR

RNA was extracted from lung tissue using a commercial kit (RNeasy Mini Kit; Qiagen, Mississauga, ON, Canada). RNA quantity and quality were assessed by using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA (cDNA) was made in a 20 μl reaction, with 250 ng of total RNA, Oligo(dT)_{12–18} primer, dNTP mix, RNaseOUT, and SuperScript[®] III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

The mRNA levels of IL-4, IL-5, IL-13 and S9 were examined. The primers were designed using Primer3 (<http://frodo.wi.mit.edu/>). The sequences of each pair of primers are shown in Table 1. Amplicons were verified on 2.5% agarose gel to be the expected size.

Quantitative real-time PCR (qPCR) was performed in 96 well plates with the Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a 10 μl reaction volume containing 0.5 μM of sense and antisense primers and 1 μl of cDNA. The plate was sealed with adhesive and the run method was

performed with three stages: holding, cycling and melt curve stage. The holding stage was 95 $^{\circ}\text{C}$ for 10 min followed by 40 cycles of 95 $^{\circ}\text{C}$ for 15 sec, annealing temperature for 30 s, and 72 $^{\circ}\text{C}$ for 30 s. The annealing temperature was 55 $^{\circ}\text{C}$ for IL-4; 60 $^{\circ}\text{C}$ for IL-5, IL-13; and 64 $^{\circ}\text{C}$ for S9. The melt curve stage was 95 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 1 min and 95 $^{\circ}\text{C}$ for 15 s. Each reaction was performed in duplicate with a negative control (RNase-free water). The comparative $\Delta\Delta\text{Ct}$ method was used for analysis, and mRNA levels of ribosomal protein S9 (reference gene) were used for normalization.

2.8. Data analysis and statistics

The data were reported as mean $\pm$ standard error and analyzed using GraphPad software (version 5). Two-way analysis of variance (ANOVA) for repeated measures was used to analyze mechanical parameters followed by Bonferroni post test. One way ANOVA with Bonferroni post test was used for the other parameters. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Intranasal challenges under halothane anesthesia induce a higher airway hyperresponsiveness than under isoflurane anesthesia

Lung mechanics are shown in Fig. 2. Halothane Sal-challenged mice had a small increase in G and H , and a reduced R_n when compared with isoflurane Sal-challenged mice ($p < 0.05$). A significant hyperresponsiveness, measured by R_n , G and H , was detected in both, isoflurane and halothane, OA-challenged mice when compared with Sal-challenged mice ($p < 0.05$). Halothane OA-challenged mice had a significantly greater increase in airway hyperresponsiveness than isoflurane OA-challenged mice, when compared with their respective Sal-challenged controls, in all parameters measured R_n , G and H , at the highest two concentrations of methacholine ($p < 0.01$). At the highest methacholine concentration, the increase (OA-challenged mice/Sal-challenged controls) of R_n , G and H was 1.75, 3.07, 2.16, respectively, in halothane OA-challenged mice and 1.39, 1.57, 1.53, respectively, in isoflurane OA-challenged mice.

3.2. The greater hyperresponsiveness observed in halothane OA-challenged mice is mostly due to a small airway involvement

The fold increase (response at the highest methacholine concentration/baseline value) of mice OA-challenged under halothane anesthesia was determined and compared to the fold increase of mice OA-challenged under isoflurane. Fold increase of G was significantly greater in halothane OA-challenged mice than in isoflurane OA-challenged mice (16.08 and 6.78, respectively, $p < 0.01$). Fold increase of R_n , and H did not reach statistical difference when halothane-anaesthetized was compared with isoflurane-anaesthetized OA-challenged mice.

Furthermore, tissue hysteresivity (η) was calculated to assess if the increase in tissue damping (G) was due to the heterogeneity of constriction rather than a real increase in tissue damping, per se (Bates and Lutchén, 2005; Bates, 2009; Lutchén et al., 1996). Differences in tissue hysteresivity did not reach statistical significance when OA-challenged mice were compared with their respective control or when halothane-challenged mice were compared with isoflurane-challenged mice (Fig. 2).

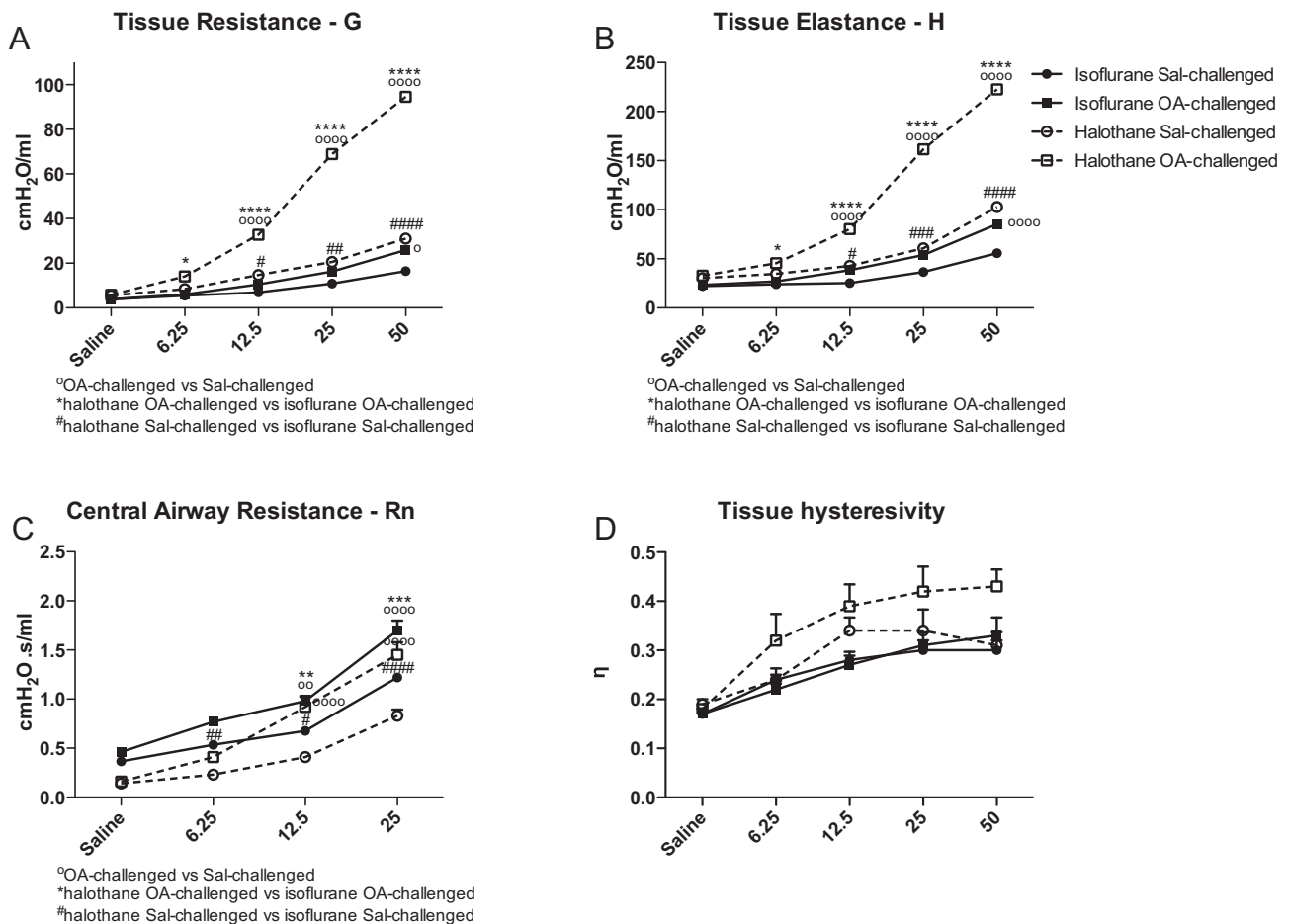


Fig. 2. Lung mechanics evaluation. Lung mechanics were evaluated 72 hours after mice were sensitized twice with OA and challenged 3 times per week for 3 weeks with OA or Sal. (A–C) A significant hyperresponsiveness, measured by Rn, G and H, was detected in both, isoflurane and halothane, OA-challenged mice when compared with their respective Sal-challenged controls. Halothane Sal-challenged mice had an increase in G and H, and a reduced Rn when compared with isoflurane Sal-challenged mice. Halothane OA-challenged mice had a higher degree of hyperresponsiveness than isoflurane OA-challenged mice, when compared with their respective Sal-challenged controls, in all parameters measured, Rn, G and H. (D) Tissue hysteresivity did not reach statistical difference among groups ($n = 7–10$). Data are presented as means $\pm$ SEM. o, * $p < 0.05$; oo, ** $p < 0.01$; ooo, *** $p < 0.001$; oooo, **** $p < 0.0001$; # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$; #### $p < 0.0001$.

3.3. Lung periphery inflammation, BAL eosinophilia and Th2 response are augmented in mice challenged under halothane anesthesia when compared to challenges performed under isoflurane anesthesia

Tissue inflammation is shown in Fig. 3. Isoflurane and halothane OA-challenged mice showed a significant increase in the number of inflammatory cells in the peribronchial area and lung periphery when compared to their respective Sal-challenged control ($p < 0.05$). Of note, halothane OA-challenged mice had a significant increase in peripheral lung inflammation when compared to isoflurane OA-challenged mice ($p < 0.01$).

Differential BAL cell counts and Th2 cytokines expression are shown in Fig. 4. The percentage of eosinophils was significantly greater in isoflurane and halothane OA-challenged mice than in their respective Sal-challenged control ($p < 0.0001$). Moreover, the percentage of eosinophils was significantly higher in halothane OA-challenged mice than in isoflurane OA-challenged mice ($p < 0.0001$). Macrophages were significantly reduced in OA-challenged mice when compared to Sal-challenged mice ($p < 0.0001$), and in halothane OA-challenged mice compared to isoflurane OA-challenged mice ($p < 0.0001$). Neutrophils and lymphocytes percentages were not different among groups.

IL-13 mRNA production was significantly increased in isoflurane and halothane OA-challenged mice compared to their control

mice ($p < 0.05$). However, IL-5 mRNA expression was significantly increased only in halothane OA-challenged mice compared to the control Sal-challenged group ($p < 0.05$) and to isoflurane OA-challenged mice ($p < 0.01$). There was no significant difference in IL-14 mRNA production among groups (Fig. 4).

3.4. Collagen deposition is increased in halothane OA-challenged mice

Collagen deposition within airway area is shown in Fig. 5. Collagen deposition was found to be significantly increased in halothane OA-challenged mice when compared to their respective Sal-challenged control ($p < 0.01$). Additionally, collagen deposition was increased in halothane OA-challenged mice compared to isoflurane OA-challenged mice ($p < 0.05$).

4. Discussion

Allergen challenges, when performed under isoflurane and halothane anesthesia, induced asthma-like characteristics in mice, including airway hyperresponsiveness, peribronchial inflammation, eosinophilia, Th2 cytokine production and airway remodeling. Mice challenged under halothane anesthesia had a more significant airway response, in terms of airway hyperresponsiveness, inflammation and remodeling, than mice challenged under isoflurane

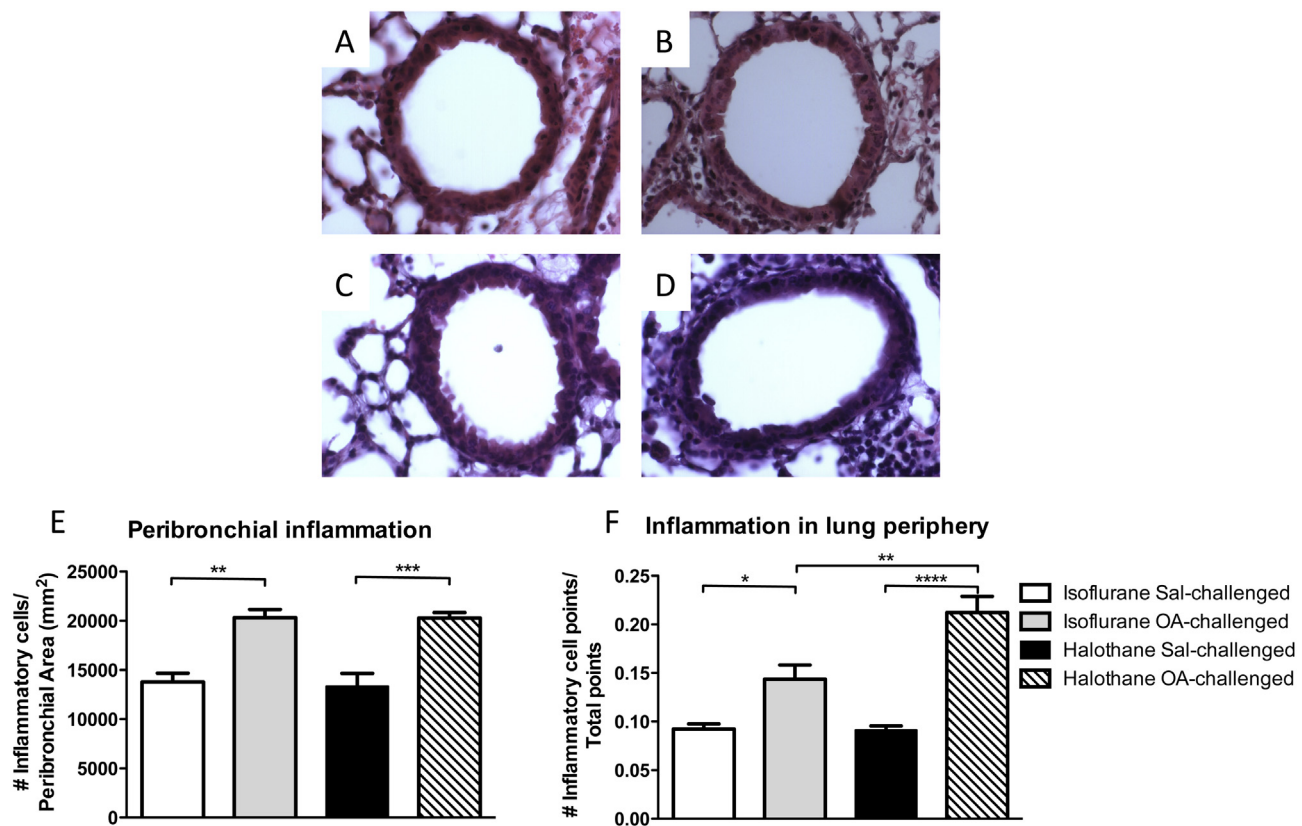


Fig. 3. Lung inflammation. (A–D) Representative lung sections stained with hematoxylin and eosin. (A) Isoflurane Sal-challenged mouse; (B) isoflurane OA-challenged mouse; (C) halothane Sal-challenged mouse; (D) halothane OA-challenged mouse. (E and F) tissue inflammation was quantified in peribronchial area and in lung periphery with the point counting method. Isoflurane and halothane OA-challenged mice showed a significant increase in the number of inflammatory cells in the peribronchial area and lung periphery when compared to their respective Sal-challenged control. Of note, halothane OA-challenged mice had an increased inflammation in lung periphery compared to isoflurane OA-challenged mice ($n = 4$ – 6). Data are presented as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

anesthesia. Considering the more potent bronchodilator action of halothane in the small airways, we postulate that mice OA-challenged under halothane anesthesia have an increased allergen deposition in the lung periphery leading to a more profound airway response.

Hyperresponsiveness is one of the defining features of asthma, and can reflect airway inflammation and/or remodeling (Cockcroft and Davis, 2006; O'Byrne and Inman, 2003). The precise mechanisms involved in hyperresponsiveness are not totally elucidated; some possibilities include reduced airway caliber, airway wall thickening, increased mucosal permeability, higher number of inflammatory cells and mediator release, structural changes, and increased airway smooth muscle contractility (Cockcroft and Davis, 2006; O'Byrne and Inman, 2003). Moreover, in the mouse model of asthma, airway closure can be a major contributor to hyperresponsiveness (Bates et al., 2008; Lundblad et al., 2007; Wagers et al., 2004).

Intranasal OA challenges performed under both anesthetic drugs, isoflurane and halothane, influenced lung mechanics. Mice OA-challenged under isoflurane and halothane anesthesia demonstrated enhanced hyperresponsiveness compared to their respective Sal-challenged controls. However, halothane OA-challenged mice had a higher increase in airway hyperresponsiveness when compared to isoflurane OA-challenged mice.

To further evaluate the contribution of different lung compartments to the airway hyperresponsiveness, we used the constant phase model that permits partitioning of the response into the large airways (R_n) and the peripheral lung compartment (G and H). The constant phase model was initially applied in dogs and,

since then, has been increasingly used in other species, such as mice and rats (Hantos et al., 1992; Pinelli et al., 2009; Shalaby et al., 2010; Siddiqui et al., 2010). The greatest airway response in the halothane OA-challenged mice was detected in G , and was significantly greater than the change observed in isoflurane OA-challenged mice. The increased airway response detected in G (tissue damping) of halothane OA-challenged mice indicates that alterations in the lung periphery were prominent and contributed significantly to the higher airway hyperresponsiveness found in this animal model. These findings are consistent with studies that show that peripheral airways account significantly for the airway hyperresponsiveness in asthma (Kuwano et al., 1993; Wagner et al., 1998; Wiggs et al., 1992; Yanai et al., 1992). These data are of particular relevance, as asthma has several clinical presentations, and heterogeneous constriction of the peripheral airways is often present (Hamid et al., 1997; Kraft, 1999).

When G increases proportionately more than H , this indicates the development of regional heterogeneities in the lung periphery. Heterogeneity increases as bronchoconstriction increases in severity (Ludwig et al., 1990; Mishima et al., 1994), consequently η may also increase and can be taken as an indicator of heterogeneity (Bates, 2009). In our results, the increase in η in halothane OA-challenged mice compared to isoflurane OA-challenged mice did not achieve significance. Hence, we believe that the increased peripheral airway response (G) found in mice challenged under halothane was not due entirely to tissue heterogeneity. Additional physiologic mechanisms that may alter G or H include derecruitment or airway closure, and changes in surfactant.

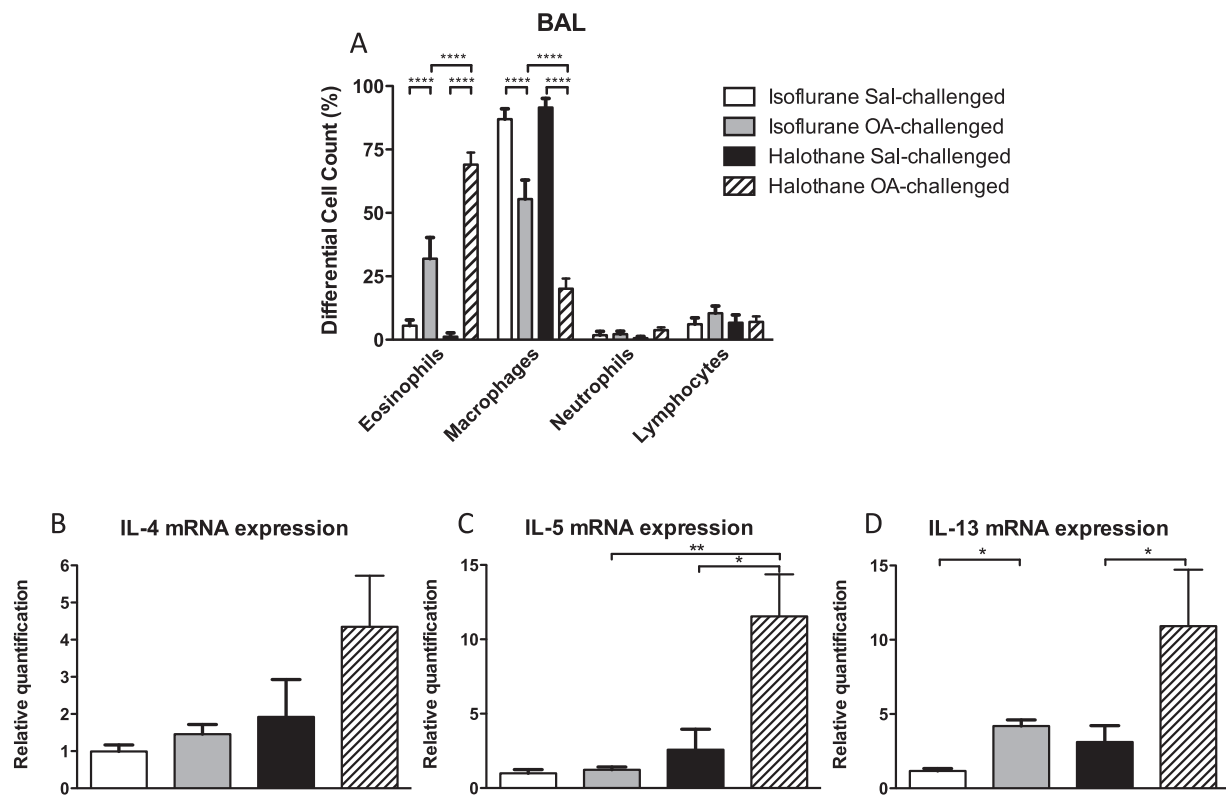


Fig. 4. Differential BAL cell counts and cytokine mRNA expression in lung tissue. (A) Differential BAL cell counts were determined by sampling three hundred cells. The percentage of eosinophils was significantly greater in isoflurane and halothane OA-challenged mice than their respective Sal-challenged control, and higher in halothane OA-challenged mice than in isoflurane OA-challenged mice. (B–D) RNA was extracted from lung tissue and quantitative real-time PCR (qPCR) was performed to quantify cytokine gene expression. IL-13 mRNA production was significantly increased in isoflurane and halothane OA-challenged mice, however IL-5 mRNA expression was significantly increased only in halothane OA-challenged mice ($n = 4-7$). Data are presented as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

The higher level of hyperresponsiveness found in mice challenged under halothane anesthesia could be related to the enhanced peripheral inflammation, Th2 response and remodeling found in this animal model. Another possible explanation could be related to an uncoupling of parenchyma and airways, due to peripheral lung inflammation (Hyde et al., 2009).

Airway inflammation, an important feature of asthma, can occur in large and small airways, and it is responsible for several of the clinical and pathophysiologic manifestations of this disease. Characteristically, there is a shift in the Th1/Th2 balance toward a Th2 response, with involvement of several cells and cytokines, such as IL-4, IL-5, IL-13 (Barnes, 2008). Several studies support a significant role of the distal lung in airway inflammation and show that the peripheral inflammatory process can exacerbate asthma, and contributes to asthma phenotype, response to treatment, accelerated lung function decline and airway remodeling (Gelfand and Kraft, 2009; Hyde et al., 2009). Biopsies from asthmatics showed a similar but more severe inflammation in the peripheral compared with the central airways (Hamid et al., 1997), and patients with nocturnal asthma have more inflammatory cells, such as eosinophils, macrophages and CD4⁺ lymphocytes, in alveolar tissue (Kraft et al., 1996, 1999).

Both isoflurane and halothane OA-challenged mice had a significant increase in inflammatory indices when compared to their respective Sal-challenged control, including an increase in the number of inflammatory cells in the peribronchial area and in Th2 cytokine production. Moreover, mice OA-challenged under halothane anesthesia had a higher number of inflammatory cells in the lung periphery than mice OA-challenged under isoflurane anesthesia. Analysis of mRNA cytokine production showed a

significant increase in IL-13 mRNA expression in isoflurane and halothane OA-challenged mice. However, IL-5 mRNA levels were significantly increased only in mice challenged under halothane anesthesia. IL-5, a Th2 cytokine, is critical for eosinophil development, survival and activation, and can contribute to hyperresponsiveness, goblet cell hyperplasia and airway remodeling (Finkelman et al., 2010; Foster et al., 1996; Humbles et al., 2004; Tanaka et al., 2004). The increased IL-5 mRNA expression found in this animal model was accompanied by a higher BAL eosinophilia and airway remodeling. Minshall et al. demonstrated an increase in IL-5 mRNA expression in the peripheral airways of asthmatics when compared with large airways (Minshall et al., 1998).

Remodeling is characterized by cellular and structural changes that lead to airway narrowing, persistent airflow limitation, decrease in lung function, and hyperresponsiveness (Al-Muhsen et al., 2011). Several factors can drive airway remodeling, such as chronic inflammation, eosinophils, epithelial–mesenchymal trophic unit, cell–cell interactions, and multiple inflammatory mediators, cytokines, chemokines, and growth factors (Al-Muhsen et al., 2011; Flood-Page et al., 2003; Holgate et al., 2010). Collagen deposition, a marker of airway remodeling, was significantly increased only in mice OA-challenged under halothane anesthesia, and this change may also have contributed to the increased airway hyperresponsiveness. Wiggs et al. have shown that moderate amounts of airway wall thickening, especially if localized in peripheral airways, can profoundly affect the airway narrowing caused by airway smooth muscle shortening, but have little effect on baseline resistance (Wiggs et al., 1992). On the other hand, Cojocaru et al., using a computational model, found no evidence that stiffness is altered in acute or chronic allergically inflamed mice

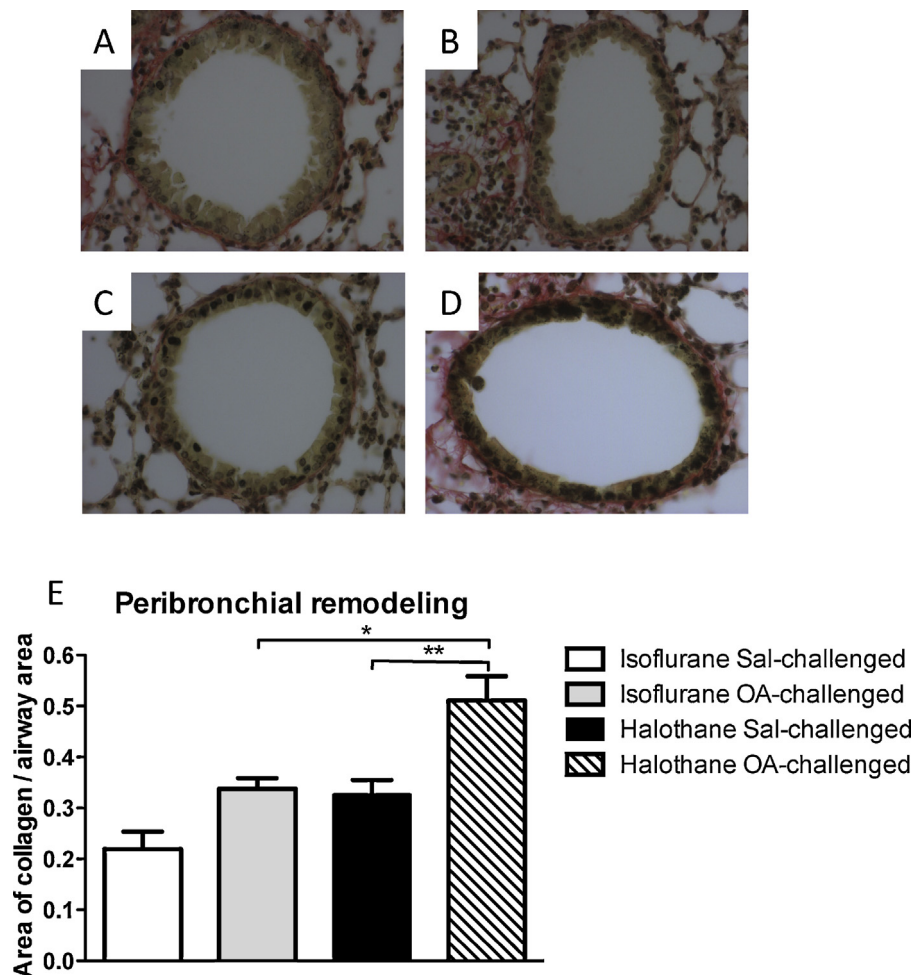


Fig. 5. Lung remodeling. (A–D) Representative lung sections stained for collagen. (A) Isoflurane Sal-challenged mouse; (B) isoflurane OA-challenged mouse; (C) halothane Sal-challenged mouse; (D) halothane OA-challenged mouse. (E) Collagen deposition was quantified in airway area as a ratio of the area of positive stain/airway area. Collagen deposition was found to be significantly increased in halothane OA-challenged mice when compared to their respective Sal-challenged control and to isoflurane OA-challenged mice ($n = 5-7$). Data are presented as means $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$.

(Cojocaru et al., 2008). The increased collagen deposition observed in this study could be a consequence of increased IL-5 mRNA production, which in turn leads to bronchoalveolar eosinophilia and consequently airway remodeling and hyperresponsiveness. Further studies are necessary to evaluate the specific effect of collagen deposition on airway mechanics and hyperresponsiveness in our model.

Aerosol airway deposition is dependent on airway caliber. Airway obstruction affects the amount of aerosol deposited in the airways and alters the deposition pattern, by increasing deposition in the central airways compared to the lung periphery (Kim et al., 1989; O’Riordan et al., 1993; Sabater et al., 2001). Conversely, bronchodilation also changes the pattern of aerosol deposition, making it more homogenous, resulting in less deposition in the central airways and enhanced deposition in the peripheral lung (Isawa et al., 1987; Saari et al., 2002). Whereas isoflurane and halothane both have bronchodilator actions, halothane is a more potent bronchodilator of the small airways (Brown et al., 1993; Mercier et al., 2002), and, consequently, could have altered allergen deposition, leading to an increase deposition in lung periphery.

Other mechanisms could have a contribution in our model, such as differences in gas density/viscosity, some local or systemic inflammatory mechanisms and/or an effect of anesthesia on basal airway tone. Isoflurane and halothane, when used in clinical concentrations, as were used in this study, only slightly affect the gas mixture viscosity (approximately 4% at most) (Habre et al.,

2001). This alteration is not likely to be physiologically significant; hence, we do not believe that the difference in the airway response could be explained by differences in gas density/viscosity. Isoflurane has been shown to have an anti-inflammatory effect (Pang et al., 2012; Reutershan et al., 2006). However, considering the very short duration of the isoflurane anesthesia used in our protocol and that the experiments were carried out 72 h after isoflurane anesthesia, we do not believe that isoflurane exerted a significant anti-inflammatory effect in the experimental conditions of the current experiment. Halothane, depending on the animal model, can have an effect on basal airway tone, by decreasing airway resistance (Reta et al., 2000; Sato et al., 1995). Considering that halothane anesthesia was used 72 h before the experiments, again, we do not believe that it could have a significant effect on the airway hyperresponsiveness of the current model.

One limitation of this study is that we did not measure the actual distribution of allergen in the airways, however the results, in terms of hyperresponsiveness and inflammation, are consistent with findings in human asthmatics in terms of the involvement of small airways. Another limitation is that we did not assess other characteristics of the immune response, such as IgE levels; further studies are necessary to evaluate the effect of isoflurane and halothane on the generalized immune response. It would also be interesting to do this in the context of different sensitization and challenge protocols.

5. Conclusions

In summary, we hypothesized that, because of the more potent bronchodilator action of halothane in the small airways, mice OA-challenged under halothane vs. isoflurane anesthesia had an increased allergen deposition in the lung periphery which induced an augmented peripheral inflammation and increased IL-5 mRNA production. This resulted in enhanced bronchoalveolar eosinophilia, airway remodeling and hyperresponsiveness, with greater involvement of the lung periphery. Our data are important in demonstrating that intranasal challenges under different anesthesia can induce different effects in airway hyperresponsiveness, inflammation and remodeling, and support the importance of the small airways in asthma pathogenesis. Such differences due to increase allergen deposition in the small airways should be considered when utilizing animal models of asthma.

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