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Am. J. Respir. Cell Mol. Biol., January 1, 2010; 42 (1): 3-8.

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Exhaled nitric oxide estimation by a simple and efficient noninvasive technique and its utility as a marker of airway inflammation in mice

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Ahmad T, Mabalirajan U, Joseph DA, Makhija L, Singh VP, Ghosh B, Agrawal A. Exhaled nitric oxide estimation by a simple and efficient noninvasive technique and its utility as a marker of airway inflammation in mice. *J Appl Physiol* 107: 295–301, 2009. First published April 30, 2009; doi:10.1152/jappphysiol.00235.2009.—Allergic airway inflammation (AI) is commonly associated with enhanced exhaled nitric oxide (ENO) in both humans and mice. Since mouse models are being used to understand various mechanisms of asthma, a noninvasive, simple, and reproducible method to determine ENO in mice is required for serial nonterminal assessment that can be used independent of environmental situations in which the ambient air contains substantial amounts of NO as a contaminant. The aim of this study was to noninvasively measure ENO in individual mice and to test its utility as a marker of AI in different models of allergic AI. We modified the existing ENO measuring methods by incorporating flushing and washout steps that allowed simple but reliable measurements under highly variable ambient NO conditions (1–100 ppb). This method was used to serially follow ENO in acute and chronic models of allergic AI in mice. ENO was reproducibly measured by this modified method and was positively correlated to AI in both acute and chronic models of asthma but was not independently related to airway remodeling. Resolution of AI and other related parameters in dexamethasone-treated mice resulted in reduction of ENO, further confirming this association. Restriction of allergen challenge to pulmonary but not nasal airways was associated with a smaller increase in ENO compared with allergen challenge to both. Hence, ENO can now be reliably measured in mice independent of ambient NO levels and is a valid biomarker for AI. However, nasal and pulmonary airways are likely to be independent sources of ENO, and any results must be interpreted as such.

inflammometry; asthma

NITRIC OXIDE (NO), a small, ubiquitous gaseous molecule in the body, is known to have significant physiological effects such as vasodilation and bronchodilation and has been implicated in the pathophysiology of inflammatory airway diseases, including bronchial asthma (4, 10, 16). It also acts as a messenger molecule in the regulation of many physiological functions such as immunity and vascular tone (5, 23). NO and other NO-adduct molecules such as nitrosothiols have been shown to be modulators of bronchomotor tone (5). A plethora of studies have revealed the multiple roles of NO in airway physiology and pathophysiology (21). NO is produced by nitric oxide synthase (NOS) in the nasal cavities, airways, and lungs and is exhaled by animals and humans. There are three distinct isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS),

and inducible NOS (iNOS). The first two are Ca^{2+} - and calmodulin dependent and release, within seconds, femtomolar or picomolar concentrations of NO upon receptor stimulation by selective agonists. On the other hand, iNOS releases large quantities (nanomolar concentrations) of NO upon stimuli such as proinflammatory cytokines (18).

Increased levels of exhaled NO (ENO) have been measured in patients with respiratory diseases such as asthma and chronic obstructive pulmonary disease (3, 11, 14, 17, 19, 20). Release of NO from inflammatory cells and activated bronchial epithelial cells may be the cause of the increased concentration of NO in the exhaled air of asthmatic patients (9, 19). The presence of NO in the exhaled air of humans and of anesthetized rabbits and guinea pigs has been demonstrated by various methods such as chemiluminescence, diazotization, and mass spectrometry. It is believed that ENO can be used as a noninvasive biomarker of pulmonary inflammation in the clinic, and its monitoring is potentially a major advance in the management of these diseases. Previous studies have measured ENO in mice and rats with either invasive approaches, such as collection of expired gas from tracheostomy tubes during mechanical ventilation (7), or noninvasive approaches, such as intermittent sampling of gas from a chamber in which a single rodent is breathing freely (15). The noninvasive approach can be considered to be either a flow-through chamber setup (24) or a sealed chamber from which a sample is taken after a predefined duration of NO accumulation (22). The first approach is technically demanding, and the second, while simple, suffers from drawbacks such as ambient air contamination at the start of measurement as well as dependence on a single point measurement. Sethi et al. (22) published data from the second method under stringent experimental conditions requiring that ambient air NO levels are <2 ppb, which in highly polluted metro cities like ours (New Delhi, India) translates to rarely or never. We present a modified method for measuring ENO that is simple and valid across a wider range of ambient conditions yet allows repetitive sampling of unanesthetized rodents so that they can be followed serially during experimental interventions. We further utilize it to investigate ENO variation related to acute and chronic models of allergic airway inflammation (AI) resembling asthma in mice.

MATERIALS AND METHODS

This study was conducted in conformity with the American Physiological Society's "Guiding Principles in the Care and Use of Animals." Experimental protocols and study design were approved by the designated institutional review boards at the Institute of Genomics and Integrative Biology.

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Exhaled nitric oxide. NO was measured with a standard clinical ENO analyzer (CLD88sp, Ecomedics) based on photometric determination of chemiluminescence, with a sampling rate of 330 ml/min, a measurement range of 0.01–1,000 ppb, and accuracy of better than 1%. Figure 1 shows the schematic layout of the experimental setup. Medical-grade NO-free air was generated by the Denox module (Ecomedics) of the analyzer at a rate of 300 ml/s. This was connected via the standard T connector used in human measurements (10-ml volume) to a Plexiglas chamber with a low volume (~450 ml; Buxco), commonly used for determination of enhanced pause (Penh) in many respiratory laboratories, such that one arm of the T was connected to the chamber and the other vented to room air. A stopcock (S1) integrated within the Buxco chamber was used to open or close the connection to the Denox module. The chamber was connected to the analyzer via a three-way stopcock (S2) such that the analyzer could rapidly be switched between ambient air and the chamber. The following sequence was followed for measurement: 1) mice were placed individually within the Plexiglas chamber; 2) the chamber was flushed with NO-free air by turning on the Denox with S1 open and the vent arm of the T connector closed; 3) chamber air was sampled to confirm zero baseline; 4) the chamber was closed by closing S1, S2 was set to sample ambient air, and the vent arm of the T was opened; 5) after 10 min, ENO recording was started and S2 was set to sample chamber air, followed immediately by opening of S1 (with vent open; withdrawal rate was set by gas analyzer flow rate, which was always 330 ml/min for every test), and an equal amount of NO free air was drawn from the T reservoir, creating a washout curve; and 6) after a stable end value was reached, recording was stopped. The initial chamber concentration (ENO_i), final chamber concentration (ENO_{eq}), and area under the curve (ENO_{auc}, mean chamber concentration × time) were recorded for each experiment. The final concentrations, representing equilibrium between the rate of NO production by the mouse and the rate of withdrawal by the analyzer, were close to zero (~1 ppb) because of the high flow rates of the analyzer (330 ml/min) that could not be adjusted in the present setup. Since washout was rigid and near complete, ENO_{auc} reflected the total amount of NO produced by the mouse over the span of the experiment. The relationship $ENO_i \times V \approx ENO_{auc} \times F$, where V is effective chamber volume and F is the sampling flow rate, was used as an internal quality control to validate the quality of the experimental data. This described the data with error of <10%.

Immunization and challenge of mice. Male BALB/c mice (8–10 wk old, weighing 18–20 g) were obtained from the National Institute of Nutrition (Hyderabad, India) and acclimatized for 1 wk under stan-

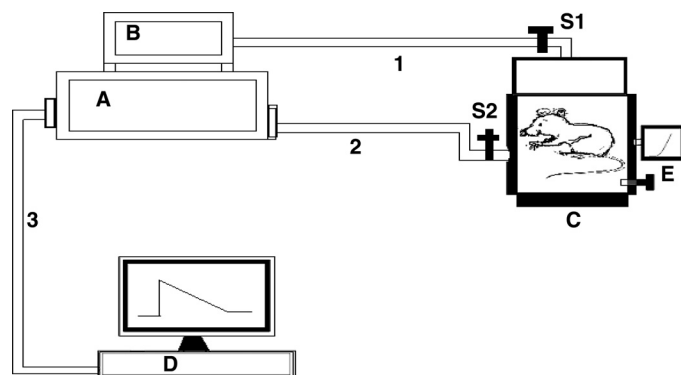


Fig. 1. Exhaled nitric oxide (ENO) measurement apparatus. A: nitric oxide (NO) analyzer. B: Denox module. C: Plexiglas chamber. D: computer. E: flow detector. These are connected with each other by the following connections: 1) Denox module connected with Plexiglas chamber via a 2-way stopcock (S1); 2) sample pipe connecting NO analyzer with the Plexiglas chamber via a 3-way stopcock (S2); and 3) data cable connecting NO analyzer with computer for online recording.

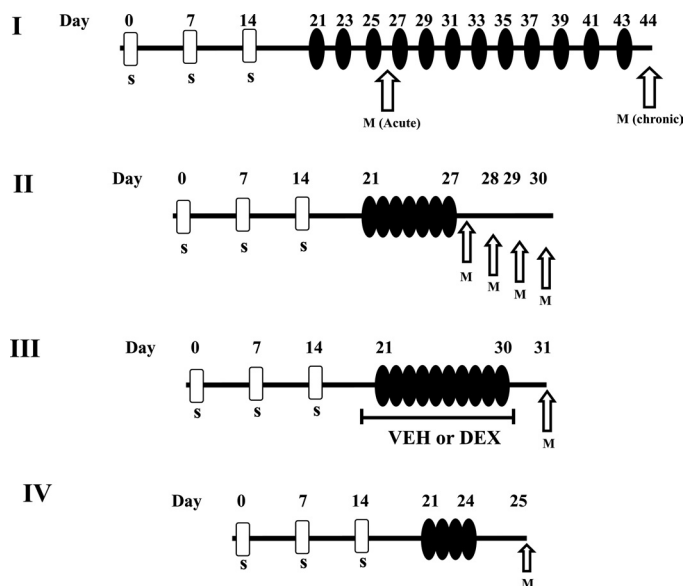


Fig. 2. Sensitization and challenge protocols. There were 4 sets of mice, and they were sensitized to ovalbumin (OVA) by 3 weekly intraperitoneal injections of 50 μ g of OVA in aluminum hydroxide (alum). One week later, set I (acute and chronic models) mice were exposed to 1% OVA aerosols on alternate days, 30 min per day, either for 1 wk (acute) or for 4 wk (chronic). ENO and airway hyperresponsiveness (AHR) were determined in set I mice on day 26 (acute) or day 44 (chronic). Set II, III, and IV mice were exposed to 3% OVA aerosols, 30 min per day, daily for 7, 10, and 4 consecutive days, respectively. ENO and AHR were determined on days 27, 28, 29, and 30 (set II, time kinetic studies), day 31 [set III, dexamethasone (Dex) experiment], or day 25 (set IV, nasal and mouth phenomena). Set III mice were administered vehicle (Veh) or dexamethasone from day 18 to day 30. All sets had Sham group mice that were sensitized with alum and exposed to phosphate-buffered saline PBS aerosols. s, Sensitization; black ovals, challenges; M, measurements of ENO and AHR.

dard laboratory conditions ($25 \pm 2^\circ\text{C}$, 55% humidity) before the experiments started. There were four sets of experiments. The protocols for all groups are shown in Fig. 2. Set I (acute and chronic models) mice were randomly divided into two groups, phosphate-buffered saline (PBS)/PBS and ovalbumin (OVA)/OVA. Later, each group was subdivided into two subgroups ($n = 6$) at the time of airway hyperresponsiveness (AHR) measurement and the time when the animal was euthanized, 1 Week and 4 Week, depending on the time of AHR measurement and the time of euthanasia. Mice were sensitized as mentioned above and challenged with 1% OVA (chicken egg ovalbumin, grade V, Sigma) dissolved in PBS for 30 min on alternate days for 1 wk or 4 wk. PBS/PBS mice were immunized with 4 mg of aluminum hydroxide (alum) and challenged with PBS alone.

Set II (time kinetic study) mice were randomly divided into two groups, PBS/PBS and OVA/OVA. Later, each group was subdivided into four subgroups ($n = 5$ –6) at the time of AHR measurement or death, 12 h, 24 h, 48 h, and 72 h, depending on the time after PBS/OVA challenge. Mice were sensitized and challenged as described previously (13). Briefly, OVA/OVA mice were immunized intraperitoneally with 50 μ g of OVA (chicken egg ovalbumin, grade V, Sigma) adsorbed on 4 mg of alum in a total volume of 200 μ l on days 0, 7, and 14. They were then challenged daily with 3% OVA dissolved in PBS for 30 min as described previously (13) for 7 days with a nebulizer with a flow rate of 9 l/min (OMRON CX model). PBS/PBS mice were immunized with 4 mg of alum and challenged with either 3% OVA or PBS alone.

Set III [dexamethasone (Dex) treatment] mice were randomly divided into three groups; each group ($n = 6$) was named according to sensitization/challenge/treatment: Sham/PBS/vehicle (Veh) (nor-

mal controls), OVA/OVA/Veh (allergic controls), and OVA/OVA/Dex (0.75 mg/kg Dex, Sigma). Dex was dissolved in absolute ethanol, a vehicle. Immunization and challenge were performed similar to those in *set I* except for a 10-day challenge. To determine the effect of Dex on baseline ENO, we also administered Dex or vehicle to nonsensitized and nonchallenged mice (dose and duration were similar to OVA/OVA/Dex).

Set IV (nasal and mouth challenge) mice were randomly divided into two groups, PBS/PBS (sham PBS challenged) and OVA/OVA. Later, each group was subdivided into three subgroups ($n = 6$) at the time of challenge such as Mouth & nose opened, Mouth only, and Nose only. Mouth & nose opened mice were challenged with both mouth and nose in open position, Mouth only mice were challenged with the nose in closed position, and Nose only mice were challenged with the mouth in closed position (2), using the nose-only configuration of the Inexpose setup (Scireq). Mice were sensitized as mentioned above and challenged with 3% OVA dissolved in PBS or PBS for 30 min for 7 days. Since sham PBS-challenged mice do not respond to PBS challenge through nose and mouth differently, they were considered identical after verification of lack of significant differences in means.

Airway hyperresponsiveness measurement. AHR to methacholine (MCh, Sigma) was determined in unrestrained and restrained conscious mice by single- and double-chamber body plethysmography, respectively (models PLY 3211 and PLY 3351, Buxco Electronics) as described previously (1, 13). Single-chamber plethysmography (SCP) was performed for the estimation of Penh, and double-chamber plethysmography (DCP) was performed for the estimation of specific airway resistance (sRaw). Despite many limitations, Penh sometimes can be useful in the BALB/c mouse model of asthma for rapid screening in a limited window of time, subject to verification by other methods. Restrained DCP was used to verify results of SCP, wherever possible, since this technique is soundly based on the first principles of physics of the lung similar to invasive techniques and allows nonterminal measurements. It has recently been shown that there is an excellent correlation between sRaw by DCP (sRaw-DCP) and airway resistance (Raw) by the forced oscillation technique (Raw-FOT) (12). Final results for SCP were expressed in MCh PC200 Penh values (the concentration of MCh required to increase the Penh to twice the baseline value) or the percent baseline Penh with each concentration of MCh, considering baseline Penh (with PBS aerosol) as 100%. Final results for sRaw-DCP were expressed in MCh PC200 sRaw values (the concentration of MCh required to increase the sRaw to twice the baseline value).

Measurements of transforming growth factor- β 1 in lung. Lung tissue homogenates in duplicate were used for transforming growth factor (TGF)- β 1 ELISA (BD Pharmingen, San Diego, CA), and results were expressed in picograms and normalized by protein concentrations.

Lung histopathology. After the ENO or AHR measurement, each mouse was killed and lungs were harvested and fixed with formalin. Fixed, paraffin-embedded, lung tissue sections were stained with hematoxylin and eosin (H & E), periodic acid, and Masson trichrome to assess AI, goblet cell metaplasia, and subepithelial fibrosis, respectively, and they were observed and microphotographs were taken with a Nikon microscope with camera (model YS-100).

Statistical analysis. Statistical significance of differences between groups was determined by ANOVA for multiple groups and with unpaired *t*-tests for two selected groups. The criterion for significance was set at $P < 0.05$. In serial measurement of ENO after allergen challenge data were analyzed by paired *t*-tests with baseline and corrected for multiple comparisons. The relationships between ENOi, ENOauc, and AI were analyzed by correlation and linear regression.

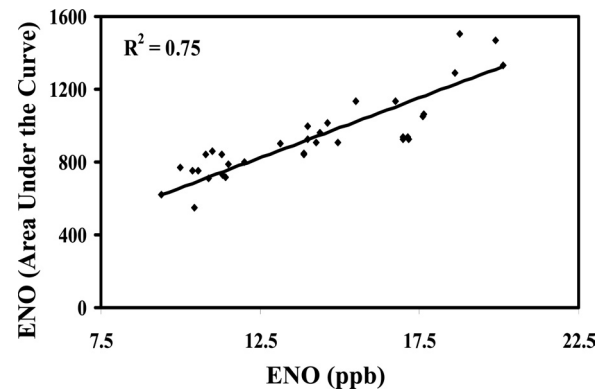


Fig. 3. Initial chamber concentration is proportional to the total area under the washout curve. Mean initial chamber concentrations are plotted against mean area under the curve (AUC) for each group of mice and fitted by simple linear regression with intercept at origin.

RESULTS

Exhaled NO levels can be noninvasively measured across range of ambient air NO conditions in simple and reproducible fashion. The experimental setup as described above was used to perform >100 ENO recordings in mice with variation of <10% between successive measurements in the same subject at the same time. As expected, the initial chamber concentration was linearly related to the total area under the washout curve across all groups (Fig. 3). The effective chamber volume, calculated from the plot based on the simplified model (see MATERIALS AND METHODS), was ~ 360 ml (slope $\times$ analyzer flow rate), which was $\sim 90\%$ of the physical volume after adjustment for the mouse and the internal plate, confirming the lack of significant leakage and validating the assumptions underlying the method. ENOi is reported exclusively for the remainder of this report since both ENOi and ENOauc were highly correlated. Notably, the ENO measurements of the Sham control mice and mice with allergic AI represent multiple measurements at various times of the day and between days with wide variations in ambient NO (1–100 ppb). No significant effect of ambient NO was observed (data not shown).

Increase in ENO correlates with AI. To study the relationship of ENO with allergic inflammation and remodeling, we measured ENOi in established murine models of allergic AI. ENOi increased from 10.6 ± 0.5 to 18.0 ± 1.7 ppb (means $\pm$ SD) after 1 wk of allergen challenge (Fig. 4A). This was accompanied by increased AI (Fig. 4B and Table 1) and AHR (Supplemental Fig. S1),¹ but there was no detectable fibrosis (Fig. 4C and Table 1), with only modest elevation of TGF- β 1 (Sham vs. acute OVA: 180 ± 33.7 vs. 490 ± 45.2 ; means $\pm$ SE). Interestingly, after 4 wk of allergen challenge, while fibrosis and TGF- β 1 were significantly greater (acute vs. chronic: 490 ± 45.2 vs. $1,016.7 \pm 20.8$; means $\pm$ SE), AI and AHR declined, accompanied by a decline in ENO. The decrease in AHR was further validated by DCP (the increase in sRaw after 60 mM MCh challenge was significantly more in the acute model: acute vs. chronic, 1.49 ± 0.22 vs. 0.44 ± 0.12 cmH₂O·s; means $\pm$ SE).

¹ The online version of this article contains supplemental material.

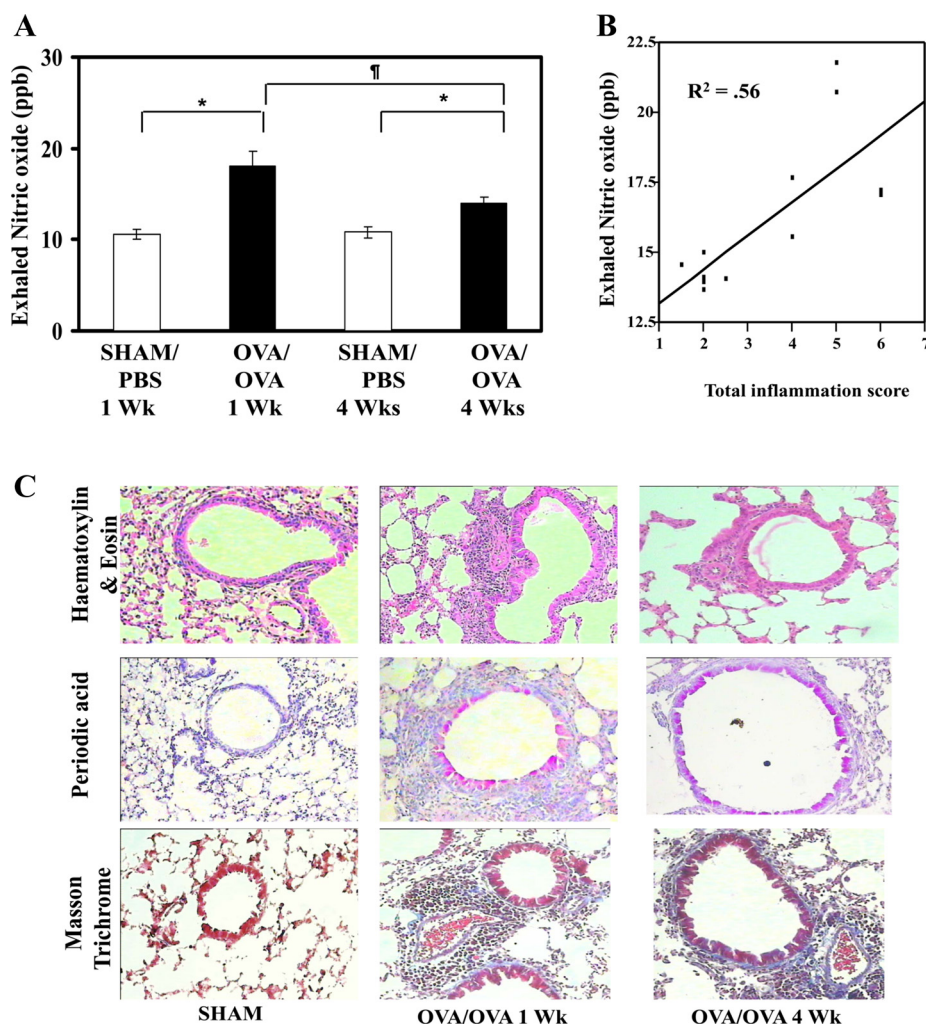


Fig. 4. Increase in ENO correlates with airway inflammation. **A**: ENO was measured as described in Fig. 1 and compared between acute and chronic models. *Statistically significant differences ($P < 0.05$). **B**: initial chamber ENO (ENOi) values of acute and chronic models are plotted against total inflammation scores and fitted by simple linear regression with intercept at origin. **C**: after the animals were killed, the lungs of mice were fixed and stained with hematoxylin and eosin, periodic acid, and Masson trichrome to estimate airway inflammation, mucous metaplasia, and subepithelial fibrosis, respectively. All photographs are at $\times 10$ magnification.

ENO rises within first 24 h after allergen challenge and is a marker of AI. Since it appeared that ENO may correlate with allergic AI, these parameters were observed at various times (12, 24, 48, and 72 h) after allergen challenge. While ENO was increased at all time points, the peak was seen by 12 h, with a decline thereafter (Fig. 5A). This correlated with the degree of

AI (Table 2, Fig. 5, B and C) and with the degree of AHR (Supplemental Fig. S2) as ascertained by SCP. To exclude the possibility that endotoxin contamination of OVA is contributing importantly to the NO response, we also measured ENOi in nonsensitized mice challenged with 3% OVA. No rise in ENO was observed in such mice compared with PBS-challenged control mice, confirming that endotoxin contamination was not contributing importantly to the increased ENO (OVA vs. PBS: 10.7 ± 0.8 vs. 10.5 ± 0.6 ppb at 12 h time point; means $\pm$ SE). Treatment with Dex resulted in the elevated AHR, AI, and ENOi returning to baseline in a parallel manner (Fig. 6 and Supplemental Table S1), supporting a link between these processes. However, Dex administration to nonsensitized and nonchallenged mice was not associated with further reductions in ENO compared with vehicle-administered mice (Dex vs. Veh: 11.0 ± 0.4 vs. 10.3 ± 0.6 ppb; means $\pm$ SE).

Allergic rhinitis may contribute to increased ENO. We recently showed (2) that, after suitable occlusion of the nasal or oral cavity, mice can breathe orally or nasally with no significant differences in minute ventilation. To investigate whether the route of challenge can impact ENO, we compared ENOi of mice subjected to allergen challenge while breathing nasally (mouth and nose open, only nose open) or orally (only mouth open). While all allergen-challenged mice had significantly

Table 1. Acute model shows more inflammation and chronic model shows more remodeling

	Inflammation Score			Morphometry	
	Perivascular	Peribronchial	Total	PAS	MT
Sham					
1 Week				5.0 ± 0.85	3.8 ± 0.28
4 Week				5.2 ± 0.25	3.3 ± 0.11
OVA					
1 Week	2.8 ± 0.22	2.4 ± 0.24	5.2 ± 0.38	15.6 ± 2.9	5.0 ± 1.1
4 Week	1.1 ± 0.08	0.9 ± 0.08	2.0 ± 0.13	12.9 ± 2.0	12.0 ± 1.58

Data are means $\pm$ SE for 3 independent experiments. Hematoxylin and eosin, periodic acid (PAS), and Masson trichrome (MT) staining were performed with lung tissue sections to assess airway inflammation, goblet metaplasia and subepithelial fibrosis, respectively. Stained slides were analyzed for inflammation score and morphometry by investigators blinded to group as described in MATERIALS AND METHODS. Sham, sham exposure; OVA, ovalbumin exposure.

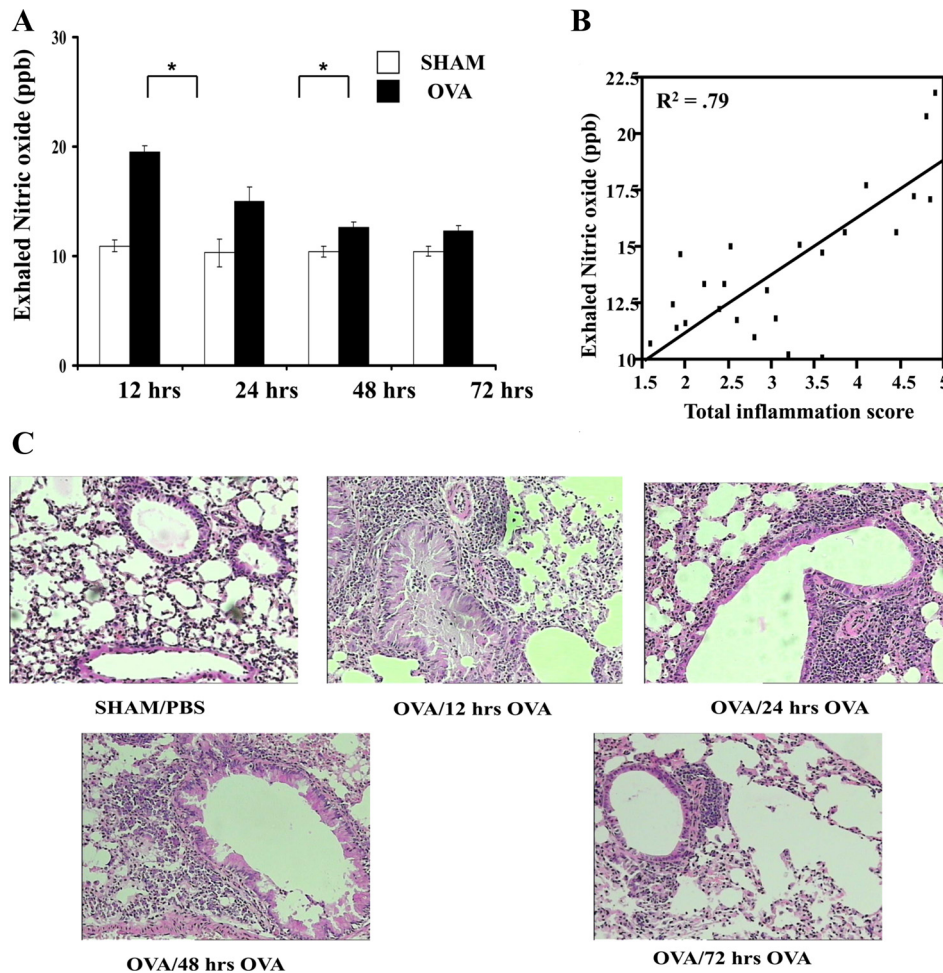


Fig. 5. ENO levels parallel with airway inflammation in time kinetic experiments. Time kinetic experiments were performed as described in MATERIALS AND METHODS. A: ENO was measured 12, 24, 48, and 72 h after last OVA exposure as described in MATERIALS AND METHODS and compared. *Statistically significant differences ($P < 0.05$). B: ENOi values from different time points are plotted against total inflammation scores and fitted by simple linear regression with intercept at origin. C: lung sections were stained with hematoxylin and eosin to estimate airway inflammation. All photographs are at $\times 10$ magnification.

higher ENOi than Sham PBS-challenged control mice, ENOi was significantly lower in mice that were challenged with allergen while breathing orally, i.e., only mouth open during allergen challenge (Fig. 7). It should be noted that measurements of ENO were made during nasal breathing. Thus the $\sim 25\%$ difference between mice that received both nasal and pulmonary allergen challenge (mouth and nose open, only nose open) and those that received pulmonary challenge (only mouth open) might reflect the nasal contribution to ENO in models of allergic AI.

DISCUSSION

We report a simplified technique for noninvasive measurement of ENO in mice that is applicable across a wide range of

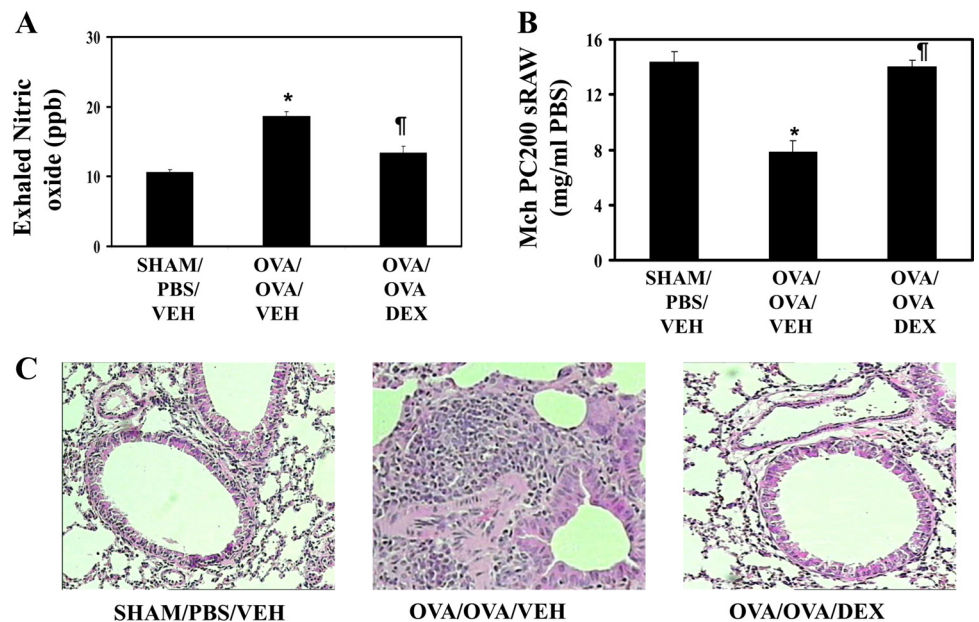
ambient NO concentrations and has an internal validation method. The measurements are highly reproducible and easy to perform, and the design is such that any of the current ENO measurement setups for clinical use can be adapted for animal research with minimal expense. There are two novel aspects to this method. First, the assembly is designed to incorporate flushing of apparatus with NO-free air in a closed configuration such that effects of changes in ambient NO levels are eliminated. Second, the design allows a controlled NO washout curve to be recorded online in real time, thereby reducing sampling error. Also, since tidal volume (V_t) and respiratory rate (RR) can be estimated in a single-chamber plethysmograph, this allows direct estimation of the average rate of NO production per expiration. While we were not able to take full advantage of the latter because of the inability to adjust inflow rate of the analyzer, an illustration is provided. In our setup with analyzer flow rates of 5.5 ml/s and mouse RR of ~ 300 breaths/min at average expiratory V_t of 0.16 ml, the observed equilibrium chamber NO concentration (NO_{Eq}) was 1.1 ± 0.4 ppb, which translates to average $\text{ENO}_{\text{mouse}}$ of $\sim 7 \pm 2$ ppb, since at equilibrium NO production equals removal ($\text{ENO}_{\text{mouse}} \times \text{RR} \times V_t = \text{NO}_{\text{Eq}} \times \text{analyzer flow rate}$). In mechanically ventilated, anesthetized, tracheostomized mice, $\text{ENO}_{\text{mouse}}$ has been reported to be 6.3 ± 0.9 ppb (7). Since we look at the NO production of nasal and pulmonary airways, the results would be expected to be somewhat higher than those in tracheos-

Table 2. Inflammation score at different time points after last OVA challenge

	Perivascular	Peribronchial	Total
12 h OVA/OVA	2.5 ± 0.13	2.2 ± 0.15	4.7 ± 0.26
24 h OVA/OVA	1.8 ± 0.16	1.5 ± 0.13	3.3 ± 0.27
48 h OVA/OVA	1.3 ± 0.11	1.0 ± 0.10	2.3 ± 0.2
72 h OVA/OVA	1.2 ± 0.12	0.8 ± 0.10	2.0 ± 0.2

Data are means $\pm$ SE for 3 independent experiments. Hematoxylin and eosin staining was performed with lung tissue sections to assess airway inflammation, and stained slides were analyzed for inflammation score by investigators blinded to group as described in MATERIALS AND METHODS.

Fig. 6. Dexamethasone reduces ENO, airway inflammation, and AHR. To determine the effect of dexamethasone, mice were sensitized and challenged and treated with dexamethasone as described in MATERIALS AND METHODS. A: ENO was measured. B: each mouse was challenged with increasing concentrations of methacholine (MCh, 0–16 mg/ml) and final results were MCh PC200 specific airway resistance (sRaw). Values are means $\pm$ SE of 3 independent experiments. *Statistically significant difference ($P < 0.05$) compared with Sham/PBS/Veh; $^{\dagger}P < 0.05$ vs. OVA/OVA/Veh. C: lung sections were stained with hematoxylin and eosin to estimate airway inflammation. All photographs are at $\times 10$ magnification.



tomized mice. However, as evidenced by the high coefficient of variation, NO_{Eq} cannot be used to precisely study between-group differences and the nasal contribution cannot be reliably estimated. The lower signal-to-noise ratio and lack of precision control in analyzer flow rates may be contributing factors. Therefore, we foresee some areas in which the method can be improved further. First, the use of an analyzer with precisely controlled low sampling flow (≤ 1 ml/s) would allow finer resolution of the washout and more precise measurement of ENO_{eq} . We do not know of any standard commercial ENO analyzer that would meet these specifications. Second, a spring-loaded nose cone could be fitted to a mouse to obtain undiluted collection of expired gas for direct measurements. Recently, a setup for DCP incorporating such a nasal cone was published in the *Journal of Applied Physiology*, and was described by the authors as leak free (12). While this is not yet commercially available,

one could visualize a future system with simultaneous measurement of sRaw and ENO.

It should be noted that although this method allows one to easily measure ENO_i under a variety of ambient conditions, the value of such measurements needed to be proven, and we have taken a step in that direction. We have found a good correlation between AHR, AI, and ENO levels. A similar correlation has been demonstrated in atopic patients (6). In fact, ENO in asthmatic patients has been considered to be useful for a noninvasive determination of AI (8). In our study, Dex treatment of asthmatic mice resulted in a reduction in the levels of ENO along with the reduction in AHR and AI (Fig. 6). Earlier studies also showed that corticosteroid treatment of asthmatic patients results in a reduction of ENO levels due to decrease of AI and inhibitory effects on iNOS expression (21). Preliminary data from our lab on iNOS expression patterns in murine models of asthma support the finding that reduction in AI is accompanied by corresponding changes in iNOS. In addition, administration of L-arginine, a NO donor, at doses of 250 mg/kg or 500 mg/kg to OVA/OVA mice has shown a significant increase in ENO levels (Mabalarajan and Ghosh, unpublished data). Thus alterations of ENO in murine models of asthma may have human correlates, and screening of ENO may be useful as a noninvasive assay with potential therapeutic efficacy while screening potential lead molecules.

The chronic allergen exposure model exhibited collagen deposition suggestive of early subepithelial fibrosis, but reduced cellular inflammation (Fig. 4C); ENO was elevated, but to a lower degree (Fig. 4A). This may be related to both the degree of cellular inflammation as well as a change in cell type. Although numerous studies suggest a role of NO in AI, only a few clarify its role in airway remodeling (9). NO has been shown to inhibit collagen synthesis in various cell types like afferent arterioles, vascular smooth muscle cells, pulmonary tissue, small intestinal lamina propria fibroblasts, and pleural mesothelial cells (23). Thus the direction of change in ENO_i does not have an unambiguous correlate in terms of alteration in pathophysiology, and further investigations are warranted.

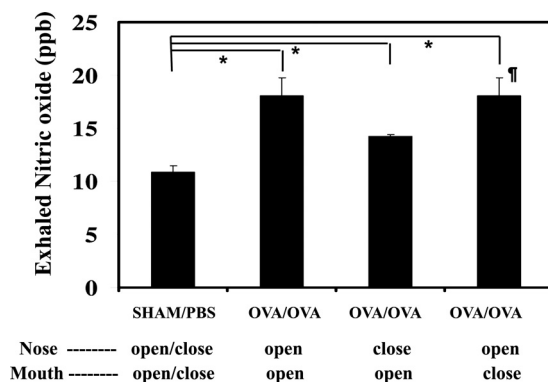


Fig. 7. Nasal inflammation contributes importantly to increase in ENO. OVA-sensitized and OVA-challenged mice (OVA/OVA) were subjected to either “nasal and pulmonary” exposure to allergen (groups with nose open) or “pulmonary only” exposure to allergen (group with nose closed), as indicated. Sham-sensitized and PBS-challenged mice did not develop inflammation irrespective of the route of exposure and are shown as a single group. Mean $\pm$ SE ENO_i is shown for each group. *Statistically significant differences ($P < 0.05$); $^{\dagger}P < 0.05$ vs. mouth only.

We also report for the first time in mice that the changes in ENO are related to the site of AI such as nasal, pulmonary, or both. We found increased ENO in mice with presumably restricted pulmonary AI and maximally increased ENO in mice with pulmonary and nasal AI (Fig. 7), suggesting an important role of inflamed nasal airways in ENO production (estimated to be ~25%). However, while it seems likely that mice challenged during oral breathing develop predominant asthma, we were unable to establish unambiguous histological correlates for allergic rhinitis in mice. Therefore, this needs further investigation. If true, it may have important implications in experimental design and data interpretation. Nevertheless, non-invasive measurement of ENO in mice is a potentially useful method to serially track AI.

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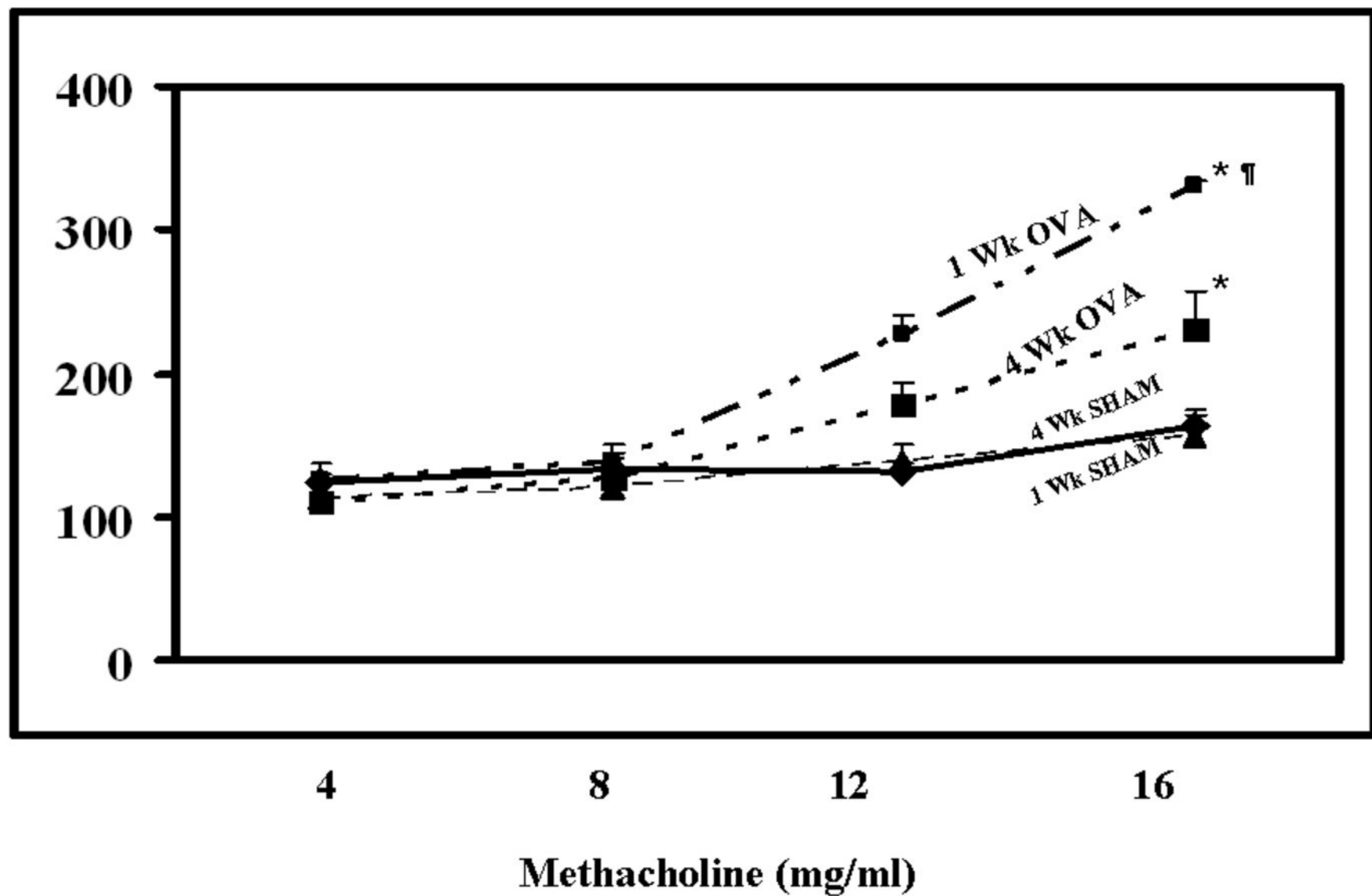
GRANTS

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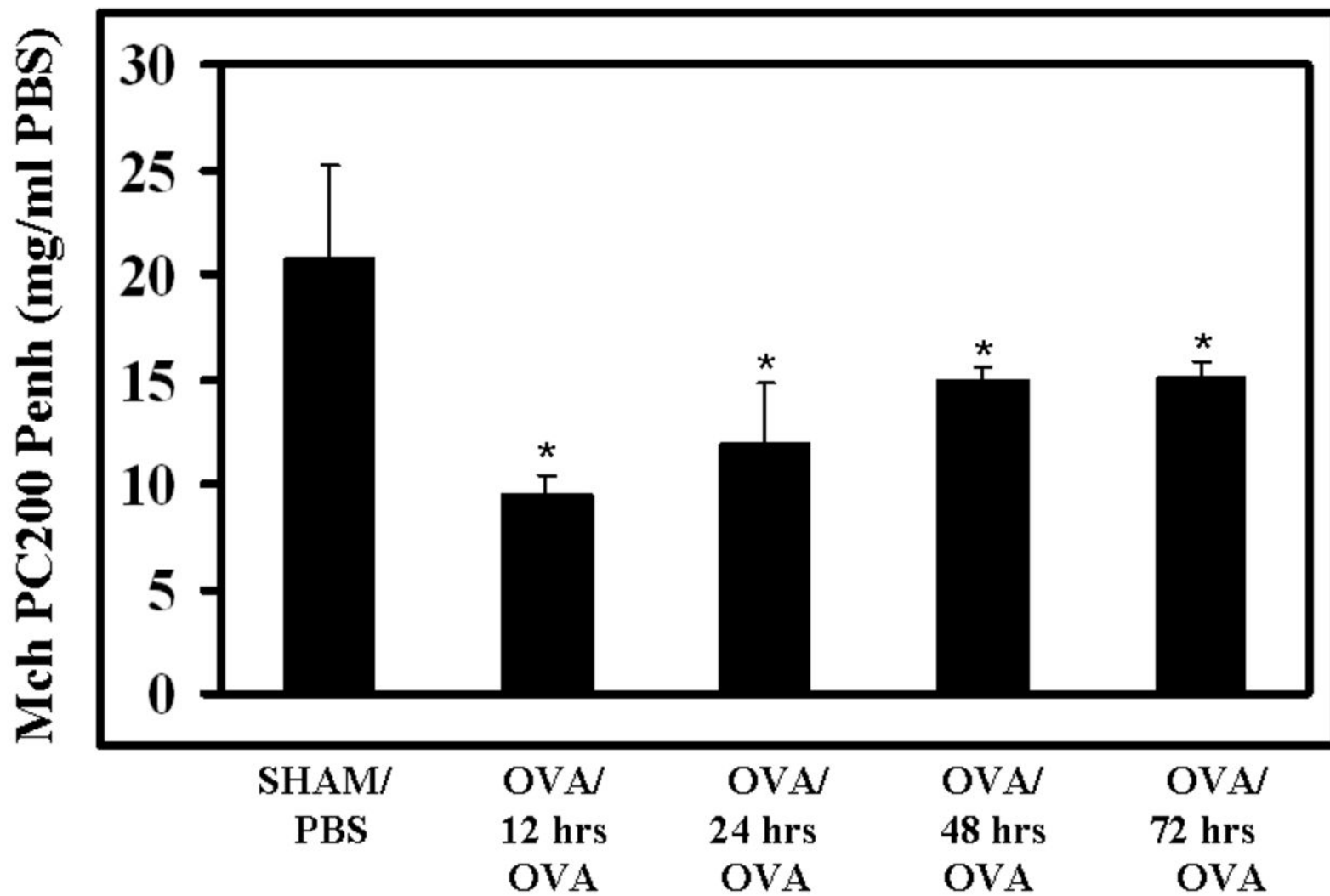
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Percentage Baseline Penh



Supp Figure 1



Supp Figure 2

Supplementary Text.**Figure legends.**

Fig. 1. *Airway hyperresponsiveness in acute and chronic model of allergic airway inflammation.* Each mouse was challenged with increasing concentrations of methacholine (0 to 16 mg/ml) and final results were expressed in percentage baseline Penh. PBS aerosol value was considered as baseline. Values plotted are mean $\pm$ SEM of three independent experiments. Further validation by sRaw measurements is reported in text.

Fig. 2. *Airway hyperresponsiveness in Time kinetic study.* Each mouse was challenged with increasing concentrations of methacholine (0 to 48 mg/ml) and final results were MCh PC200 Penh. Values plotted are mean $\pm$ SEM of three independent experiments. * denotes statistically significant difference ($p < 0.05$) compared to sham controls.

Table 1. *Dexamethasone reduces airway Inflammation.*

	Inflammation score		
	Peri-vascular	Peri-bronchial	Total
SHAM/PBS/OVA	---	---	---
OVA/OVA/VEH	3.8 ± 0.07	3.2 ± 0.15	6.7 ± 0.20
OVA/OVA/DEX	0.9 ± 0.12	0.7 ± 0.07	1.6 ± 0.18

Haematoxylin & Eosin staining was performed with lung tissue sections to assess airway inflammation and stained slides were analyzed by blind investigators for inflammation score as described in *Materials and Methods*. Data were mean ± sem three independent experiments.