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Ryan Vaughan

*University of Connecticut - Storrs*, [ryan.vaughan84@gmail.com](mailto:ryan.vaughan84@gmail.com)

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## Potential Role of Adenosine Signaling in Acetic Acid Activation of Murine Sensory Neurons

Ryan Vaughan

**Abstract**

Chronic respiratory illnesses are a significant cause of morbidity and mortality, and acute changes in respiratory function often lead to hospitalization. Air pollution is known to exacerbate asthma, but the molecular mechanisms of this are poorly understood. The current studies were aimed at clarifying the roles of nerve subtypes and purinergic receptors in respiratory reflex responses following exposure to irritants. In C57Bl/6J female mice, inspired adenosine produced sensory irritation, shown to be mediated mostly by A $\delta$  fibers. Secondly, the response to inhaled acetic acid was discovered to be dually influenced by C and A $\delta$  fibers, as indicated by the observed effects of capsaicin pretreatment, which selectively destroys TRPV1-expressing fibers (mostly C fibers) and pretreatment with theophylline, a nonselective adenosine receptor antagonist. The responses to both adenosine and acetic acid were enhanced in the ovalbumin-allergic airway disease model, although the particular pathway altered is still unknown.

*Key Words: sensory irritation; acetic acid; adenosine; capsaicin.*

**Introduction**

The ability to respond to inhaled chemicals is a crucial survival mechanism to protect against both damage to the airways and systemic exposure to dangerous substances. The respiratory tract is a large and complex system, innervated with nerves from multiple

sources. Airborne pollutants that stimulate nasal trigeminal sensory neurons are called sensory irritants. In healthy subjects, inhaled irritants may cause irritation or nothing at all. Subjects who are exercising have significantly increased tidal volumes and minute volumes and thus, their total exposure to irritants is increased as well. In individuals with allergic airway disease (allergic rhinitis, asthma, or reactive airway disease), such irritants may exacerbate disease. Unfortunately, the precise mechanisms through which irritants stimulate sensory nerves in healthy individuals or in individuals with allergic airway disease are not known.

Airborne acidity is but one category of irritants, but it is well known for producing respiratory changes. Naturally-occurring acidic fog has been shown to be quite acidic and detrimental. In Kushiro, Japan, the average pH of fog from 1992 to 1995 was under 5, with a minimum of 2.8 (Honma 2000). Hospital visits increased by 8.8% on fog days, fog was the most important contributing factor to the increase in visits compared to other meteorological and air pollutant values, and only 19.5% of patients surveyed reported no exacerbation of asthma on foggy days.

An observational study in Denver, Colorado throughout the winter of 1987-88 surveyed 207 asthmatic adult patients. The researchers evaluated sulfates, nitrates, particulate matter < 2.5 microns in diameter (PM<sub>2.5</sub>), sulfur dioxide, nitric acid, hydrogen ion, and minimum temperature. The study participants kept diaries including the presence and severity (0 = none to 4 = incapacitating) of cough, wheeze, shortness of breath, chest tightness, and sputum production, as well as physician and emergency department visits. The researchers also gathered information related to frequency of medication use, time spent outdoors, levels of exercise intensity and whether exercise occurred indoors or outside,

indoor exposure to respiratory irritants (such as gas stoves, fireplaces and environmental tobacco smoke), and occupational exposures. Out of all these measures, the authors found that only exposure-adjusted hydrogen ion levels were associated with the probability of reporting both a moderate or worse cough and with worsening asthma rating (Ostro 699).

Although the effects of inhaled acids are readily apparent from these observational studies, the mechanism or mechanisms through which these effects occur is not as straightforward. The relevant science involved is reviewed briefly below.

There are two basic types of sensory neurons: A $\delta$  fibers and C fibers. A $\delta$  fibers are myelinated, rapidly conducting neurons ( $5\text{-}25\text{ m s}^{-1}$ ) that tend to respond to low stimuli thresholds, such as temperatures below 5 degrees Celsius or, in another organ system, injury to the skin. The slow-conducting, non-myelinated C fibers innervating the airways conduct action potentials less than  $1.5\text{ m s}^{-1}$  and are referred to as nociceptors because they tend to not respond to the mechanical forces occurring during normal respiration. Instead, they respond primarily to noxious or potentially tissue-damaging stimuli (Kollarik 2003). C fibers also respond to temperatures above 45 degrees Celsius as well as chemical, and mechanical stimuli. The chemical stimulus for such nociceptors may be exogenous or endogenous.

Past research has focused on the vanilloid receptors, now known as the transient receptor potential vanilloid receptor (TRPV1), a ligand-gated ion channel that is expressed by some C fibers. It is activated in response to low extracellular pH, vanilloid receptor ligands such as capsaicin, or heat in excess of  $\sim 42\text{-}45$  degrees Celsius. There is also a similar TRPV2 receptor, which responds to heat only above 50 degrees Celsius. Capsaicin has been shown to stimulate C fibers via the TRPV1 receptor; Kollarik *et al* (2003) showed that 90% of C fibers in the *in vitro* murine isolated/perfused lung-nerve preparation responded to

capsaicin, whereas none of the isolates from TRPV1<sup>-/-</sup> animals responded. The TRPV1 receptors are found much more commonly on C fibers compared to A $\delta$  fibers, and so C fibers respond to capsaicin preferentially more than A $\delta$  fibers. At higher doses, capsaicin causes a prolonged degeneration of TRPV1-expressing sensory nerves (Morris *et al.*, 2003). In addition, the TRPV1 antagonists capsazepine and iodo-resiniferatoxin have been shown to inhibit activity of the C fibers normally produced by low dose capsaicin and acidity (Fox 1995).

Kollarik (2003) also showed that C fibers in the *in vitro* murine isolated/perfused lung-nerve preparation responded to acid (pH 5) in both wild-type TRPV1<sup>+/+</sup> and knockout TRPV1<sup>-/-</sup> mice. The number of action potentials generated was halved in the TRPV1<sup>-/-</sup> group, but not abolished. However, the response was not present in a TRPV1<sup>+/+</sup>, but capsaicin-insensitive preparation. These results suggest that the TRPV1 receptor is not required for the activation of C fibers by acid, but that capsaicin-sensitive fibers do participate in the response. This turned our attention toward alternate receptors and mechanisms for acid-induced sensory nerve irritation.

Purines are heterocyclic aromatic organic compounds containing a pyrimidine ring fused to an imidazole ring. The purine receptors P2X and P2Y respond to the binding of adenosine triphosphate (ATP). The P2X receptor is a ligand-gated ion channel whereas the P2Y receptor is a G-protein coupled receptor. In addition, ATP is degraded by ectoenzymes to ADP, AMP, and adenosine. *In vivo* ATP likely acts both directly on P2 receptors and, following degradation to adenosine, acts on adenosine (A) receptors (see Figure 4, Vaughan *et al* 2006).

The lower respiratory tract, including the trachea, bronchi, bronchioles, and the rest of

the lungs, is innervated by the vagus nerves, parasympathetic nerves that also innervate the heart, kidneys, and gastrointestinal system down to the proximal colon. Kollarik and Dinh *et al* (2003) showed that the vagus nerves can be stimulated by P2X receptor agonists in mice. The trigeminal nerves innervate the upper respiratory tract (URT), which consists of the larynx and oro- and naso-pharynxes and the nasal cavity. It has not been shown whether the trigeminal nerves of mice respond to stimulation by purines.

Stimulation of receptors in the respiratory tract likely produces both local and central effects. Possibly depending on the cause of the stimulation, some of these effects may be inflammation, vasodilation and edema, mucus secretion, airway obstruction, cough, and changing breathing patterns. Sensory irritants are defined as compounds that stimulate nasal trigeminal C fibers and decrease respiratory rate by producing breath-holding, or prolonged pauses at the start of expiration. Alaire has implicated the trigeminal nerve in these effects by showing that trigeminal nerve ligation eliminates this response in mice (Alaire 1973). In animal models, the sensory irritation response is characterized by “braking” at the onset of the expiratory phase.

One explanation for the sensory irritation response is that irritants directly stimulate sensory neurons. So-called “irritant receptors” would have to exist separately for every possible irritant, or the receptors would have to be non-specific enough to respond to a broad diversity of irritants. Another possibility is that irritants cause the release of some paracrine signal, which then interacts with the particular receptors on the neurons. A system like this would require far fewer receptor types to respond to any cellular injury from irritants, and this model is not unprecedented. Bertrand (2003) described the role of purinergic signaling in the enteric nervous system. Zhong *et al* (2003) described the P2X receptor expression on

sensory neurons in rat bladders. More recently Ahmad *et al* (2005) showed that human and rat lung epithelial cells release ATP during exposure to ozone. Furthermore, artificial removal of the ATP and adenosine potentiated ozone toxicity, suggesting a protective role for the ATP.

The aims of the present studies were multifold: 1) to better characterize the role of C fibers vs. A $\delta$  fibers across a range of acetic acid concentrations and in diseased (OVA) mice, 2) to determine which of either nerve type was most sensitive to acetic acid, and 3) to determine the role of adenosine signaling itself in this process. To test the hypothesis that adenosine signaling is involved in the response to acetic acid, numerous experiments were performed. Mice were exposed to adenosine with and without capsaicin-pretreatment or the A1-selective receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Then, mice were exposed to acetic acid with and without capsaicin pretreatment or the non-selective A receptor antagonist theophylline. Finally, control mice and OVA-AAD mice were exposed to adenosine or acetic acid to determine the magnitude of changes in responsiveness in disease. The most prevalent atmospheric acids are sulfur dioxide, sulfuric acid, and nitric acid; in the current studies acetic acid was chosen as a representative acid because it has been previously studied in our laboratory.

## **Materials and Methods**

*Animals and exposure methodology:* C57Bl/6J female mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were used in all studies. Animals were 5-6 weeks of age at purchase and were housed over hardwood shavings (Sani-Chip Dry, P.J. Murphy Forest Products, Montville, NJ) in animal rooms maintained at 22-25°C with a 12

hour light-dark cycle (lights on at 6:30 am). Food (Lab Diet, PMI Nutrition International, Brentwood, MO) and tap water were provided *ad libitum*. Animals were acclimated for at least 1 week prior to use and were used within 8 weeks of arrival. Body weights averaged ~20 grams at the time of use. All protocols were approved by the University of Connecticut Institutional Animal Care and Use Committee.

**Experimental Designs:** The initial experiments were intended to decipher the relative roles of capsaicin-sensitive nerves and A1 receptors through the use of inhaled adenosine, the A1 receptor-selective antagonist DPCPX, and capsaicin pretreatment. Next, the potential roles of capsaicin-sensitive (primarily C) fibers versus capsaicin-sensitive (primarily A $\delta$ ) fibers in the response to acetic acid were addressed. To further elucidate the role of A receptors in the response to inhaled acetic acid, acetic acid was given with and without the non-selective A receptor antagonist theophylline. To determine if sensory nerve responsiveness was altered in allergic airway disease, the response to adenosine aerosol was examined in mice with OVA-AAD using the protocols previously published and described below. And finally, the response to acetic acid in mice with OVA-AAD was examined.

**Exposure Protocols:** As in our previous studies (Vaughan *et al* 2006), spontaneously breathing mice were exposed and respiratory parameters monitored in a Buxco double plethysmograph using the Buxco noninvasive mechanics software (Buxco, Inc., Sharon, CT). Animals were restrained in the plethysmograph by a latex collar, but were not anesthetized. Multiple respiratory parameters were analyzed including breathing frequency, tidal volume, time of inspiration and expiration, peak flows during inspiration and expiration, the duration of braking in early expiration, and the duration of any pause at the end of expiration. Because sensory irritation is characterized by braking at the onset of expiration (Alarie 1973), the

current study focused on this parameter. Clean or irritant laden air was drawn into the headspace of the double plethysmograph at a flow rate of 0.6 L/min. Air temperature ranged between 22 and 25°C and relative humidity averaged 50%. After a 10 minute acclimatization period, a 10 minute baseline exposure to clean air commenced. This was followed by the 15 minute experimental exposure to irritant. Breathing parameters were collected throughout the time the animal was in the plethysmograph. One-minute average values were recorded and used for statistical analysis as is typical for sensory irritation protocols. Plethysmograph headspace air samples were drawn during exposure and analyzed for irritant concentration. Gas chromatography or high-performance liquid chromatography was used for this, depending on the nature of the gas or aerosol.

***Drug Protocols:*** The adenosine receptor antagonist theophylline was prepared as a 5 mg/ml solution in saline. It was administered via intraperitoneal injection at a dose of 20 mg/kg 20-30 minutes before aerosol exposure. The A1 receptor antagonist DPCPX was prepared as a 0.01 mg/ml solution in 2% dimethylsulfoxide (DMSO) in saline. It was given via intraperitoneal injection at a dose of 0.1 mg/kg 20-30 minutes before aerosol exposure. Capsaicin pretreatments were performed by the protocol used previously, as follows. Animals received a subcutaneous injection of capsaicin at 25 mg/kg followed by 75 mg/kg two days later. The capsaicin was 5 mg/ml, dissolved in a 1:1:8 solution of ethanol:Tween80:saline. Prior to each injection, animals were anesthetized with intraperitoneal avertin at 250 mg/kg and then treated with subcutaneous theophylline (10 mg/kg, 5 mg/ml in distilled water) and intraperitoneal terbutaline (0.1 mg/kg, 0.05 mg/ml in saline) to minimize respiratory side effects. Control mice received a vehicle injection and the avertin, theophylline, and terbutaline. Responsiveness to capsaicin aerosol challenge is

markedly reduced for at least 5 weeks by this protocol (Morris *et al* 2003, Symanowicz *et al* 2004) but animals were used 1-2 weeks after treatment.

OVA-AAD was induced by the protocol used previously, as follows. Animals received three weekly intraperitoneal injections of 25 micrograms OVA (Grade V, Sigma Chemical Company, St Louis, MO) adsorbed to 2 mg aluminum hydroxide. One week after the last injection, animals were exposed for 1 hour per day for eight days to aerosolized OVA in a directed airflow nose-only exposure chamber (CH Technologies, Westwood, NJ). Atmospheres were generated by nebulization (Lovelace Nebulizer, In-Tox Products, Albuquerque, NM) of 1% OVA in saline. Airborne OVA concentrations averaged ~20 mg/m<sup>3</sup>. Control animals received the intraperitoneal OVA injections but no OVA aerosol. Responsiveness to adenosine was measured 1 day after the eighth daily exposure.

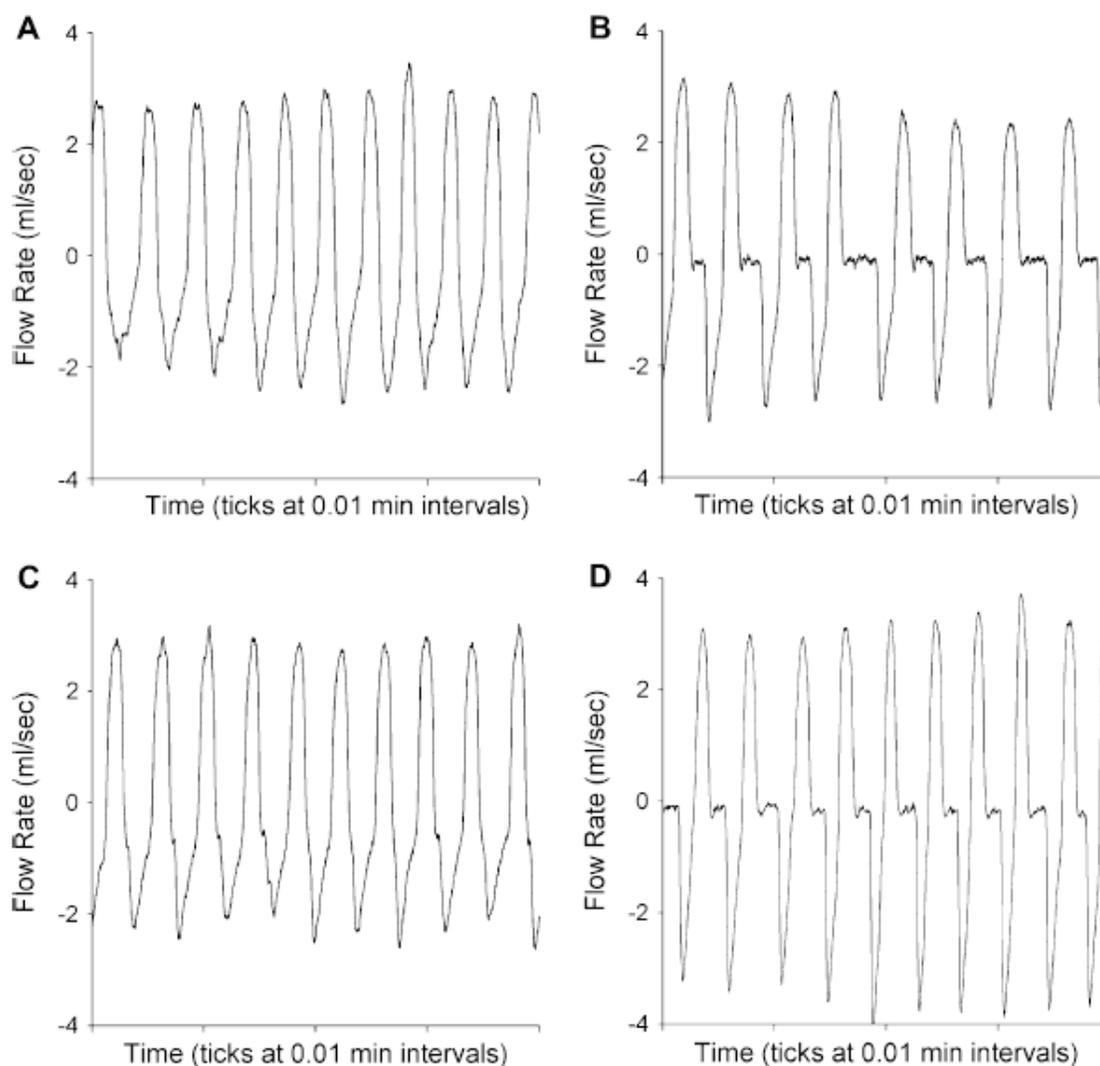
***Irritant Generation and Analysis:*** Adenosine aerosol was generated with a Lovelace nebulizer. Particle size averaged 1.1  $\mu\text{m}$  MMAD ( $\sigma = 2.1$ , Mercer impactor). Nebulization solutions contained 10  $\mu\text{g/ml}$  fluorescein as a tag. During exposure headspace air was drawn through a 0.2- $\mu\text{m}$  filter, the filter was eluted with 10mM NaOH, and fluorescence determined. Airborne concentrations were calculated stoichiometrically from the fluorescein tag data. Acetic acid was obtained from Fisher Scientific. Acetic acid vapor was generated by flash evaporation. Plethysmograph headspace samples were drawn through two midget impingers in series, each containing 10 ml distilled water. The connecting tubing was flushed with 1 ml distilled water following exposure. The concentration of acetate in the impinger fluid was determined via high-performance liquid chromatography with ultraviolet detection at 210 nm (Varian Model 2510) using a mobile phase of 5:95 vol:vol acetonitrile:0.1% H<sub>2</sub>PO<sub>4</sub> at a flow rate of 1 ml/min.

**Data Analysis:** Data are presented as mean  $\pm$  standard error of the mean (SEM) unless otherwise indicated. Expiratory braking data were collected as 1 minute averages in each animal during the 10 minute baseline and 15 minute exposure periods. Data were log transformed as appropriate to correct for heteroscedasticity. A repeated measures of variance (ANOVA) followed by Newman-Keuls test was performed on each animal group (with the repeated measure being time) to determine if expiratory braking duration during exposure differed significantly from baseline levels as revealed by a significant effect of time. To determine if the pharmacological agents altered the responses, expiratory braking data obtained during the exposure periods only were compared by two-factor repeated measures ANOVA with one factor being drug pretreatment and the other factor being the repeated measure, time. Each animal was exposed only once, thus these represent between-animal comparisons. These comparisons often revealed a statistical interaction between drug pretreatment and time. If so, individual comparisons among drug groups were made at each exposure time by ANOVA. All statistical calculations were performed with Statistica Software (StatSoft, Tulsa, OK). A  $p < 0.05$  was required for significance.

### **Results:**

Characteristics of the general responses to airborne chemicals has been described previously (Vijayaraghavan *et al.*, 1993). Figure 1 shows the digitized flow signals for a typical mouse exposed to ATP in our lab. During baseline no braking was observed at early expiration (Figure 1A). During the first minute of exposure to the ATP (Figure 1B), braking was observed at the onset of each expiration, a pattern characteristic of sensory irritation (see Fig. 4D in Vijayaraghavan *et al.*, 1993). There was no apparent pause at the end of expiration

suggesting that pulmonary irritation did not occur. Breathing patterns appeared similar to baseline by minute 5 of exposure (Figure 1C) and remained similar to baseline throughout the rest of exposure. The response to adenosine during the first minute of exposure (in a separate mouse) is shown for comparison in Figure 1D. There was an induction of braking at the onset of expiration without apparent alteration in other aspects of breathing pattern, a response pattern similar to that seen for ATP in Figure 1B.



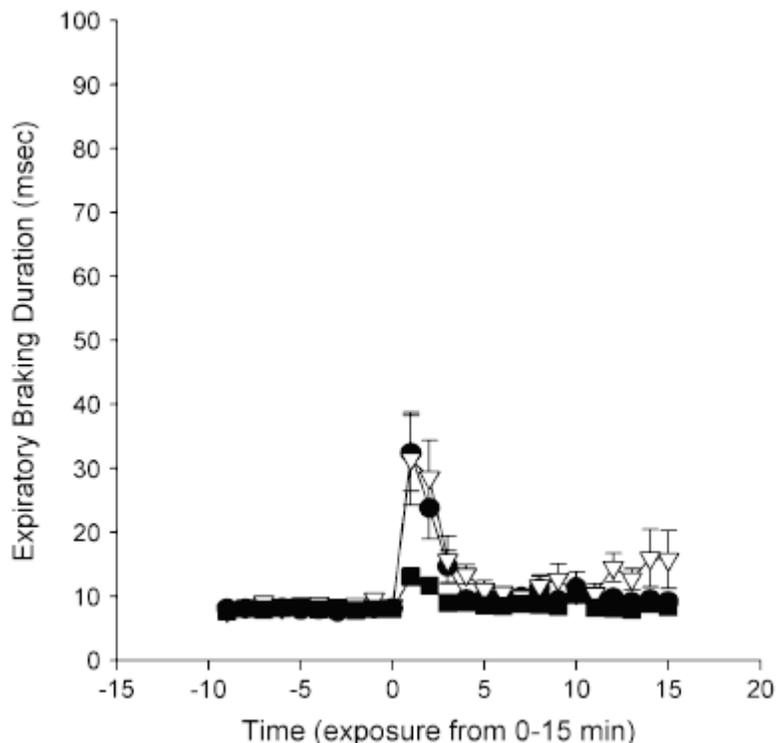
**Figure 1:** Shown are digitized respiratory flow signals obtained from a typical mouse during baseline (A), minute 1 (B), and minute 5 (C) of exposure to ATP, as well as for minute 1 (D) of exposure to adenosine from a separate animal for comparison. The zero point on the vertical axis separates inspiration (positive, upward) from expiration (negative, downward). A prolonged braking was seen at the onset of expiration during minute 1 of

exposure but not during the baseline or during minute 5. The response patterns during minute 1 of exposure to ATP (B) and adenosine (D) were similar. Each tick mark on the horizontal time axis represents 0.01 min.

### *Adenosine Sensory Irritation: Role of Capsaicin-Sensitive Nerves and A1 Receptor Pathways*

To explore the role of sensory nerves in the response to adenosine, two pretreatments were given. Capsaicin pretreatment, following the protocol outlined above, was given to one group of mice to selectively destroy TRPV1-expressing fibers. To assess the potential role of the A1 receptor in mediating responsiveness, DPCPX, a selective A1 receptor antagonist, was used to block the effects mediated by A1 receptors. Figure 2 shows the response to 14 mM adenosine by control, capsaicin, and DPCPX groups. Airborne adenosine concentrations averaged 95 nmol/L. The exposure groups contained 5-12 mice. The control mice received either capsaicin vehicle injection 1-2 weeks prior to exposure or no pretreatment, and the data were pooled to form a single control group after statistics showed no difference.

Baseline breathing frequency and minute ventilation were similar in all groups and averaged  $303 \pm 26$  breaths/min and  $85 \pm 10$  ml/min, respectively. Adenosine induced a mild transient expiratory braking response in the control mice, with the peak response being 32 ms. In the DPCPX-pretreated mice, the peak expiratory braking duration response to adenosine was 13 ms. In capsaicin-pretreated mice, the peak response was 31 ms. Comparison of the response data by two-factor ANOVA revealed a significant difference among groups ( $p < 0.05$ ), and a significant effect of time ( $p < 0.001$ ). A statistically significant interaction between these factors was not detected. Newman-Keuls test revealed that the response in the control and capsaicin-pretreated mice were statistically similar ( $p > 0.05$ ) and the response in the DPCPX-pretreated mice was significantly ( $p < 0.05$ ) lower than that in both other groups.

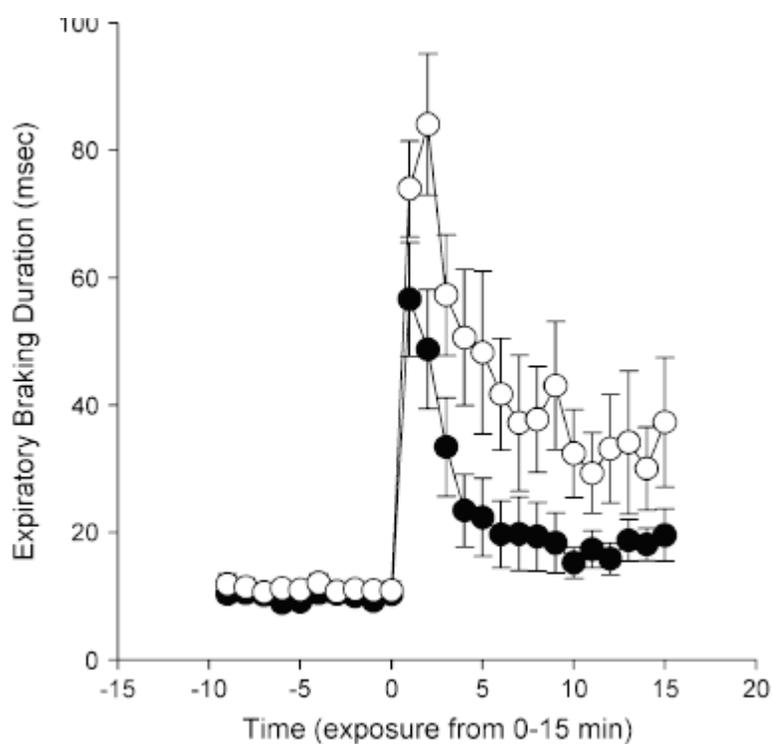


**FIGURE 2:** Shown are the average expiratory braking durations during each minute of baseline (minutes –9 to 0) and aerosol exposure (minutes 0 to 15) to 14 mM adenosine in control mice (closed circles), mice pretreated with capsaicin (open triangles), and mice pretreated with DPCPX (closed squares). Repeated measures ANOVA revealed that expiratory braking duration was significantly increased over baseline in minutes 1-3 in control mice and minutes 1 and 2 in capsaicin- or DPCPX-pretreated mice. Two-factor ANOVA revealed a significant difference among pretreatment ( $p < 0.05$ ), a significant effect of time ( $p < 0.0001$ ) and no interaction between these factors ( $p > 0.05$ ). Newman-Keuls test revealed that the response in the DPCPX group was significantly lower than in control ( $p < 0.05$ ) and that the response in the capsaicin and control groups did not differ significantly ( $p > 0.05$ ). Data are presented as mean  $\pm$  SEM; the groups contained 5-12 mice.

#### *Adenosine Sensory Irritation: Enhancement in Allergic Airway Disease*

Shown in Figure 3 is the response to 14 mM adenosine aerosol in OVA-d0 (control) and OVA-d8 mice. Airborne adenosine concentrations average 115 nmol/L. Exposure groups contained 8-10 mice. In OVA-d0 mice, expiratory braking duration was significantly elevated over baseline levels in minutes 1-4 of exposure. In OVA-d8 mice, expiratory braking duration was significantly elevated throughout the entire exposure with the overall response being approximately two-fold greater than in healthy mice. Two-factor ANOVA

revealed a significant difference between OVA-d0 and OVA-d8 ( $p < 0.05$ ). A statistical interaction between time and ovalbumin treatment was not detected. Baseline breathing frequency was similar in control and OVA-d8 animals and averaged  $286 \pm 25$  (mean  $\pm$  SD). Minute ventilation averaged  $73 \pm 9$  ml/min in control versus  $63 \pm 6$  ml/min (mean  $\pm$  SD) in OVA-d8. These values were significantly different ( $p < 0.02$ ), indicating that the OVA-d8 mice received a lower inspired dosage than the control mice due to the reduced minute ventilation, yet still produced a greater response.

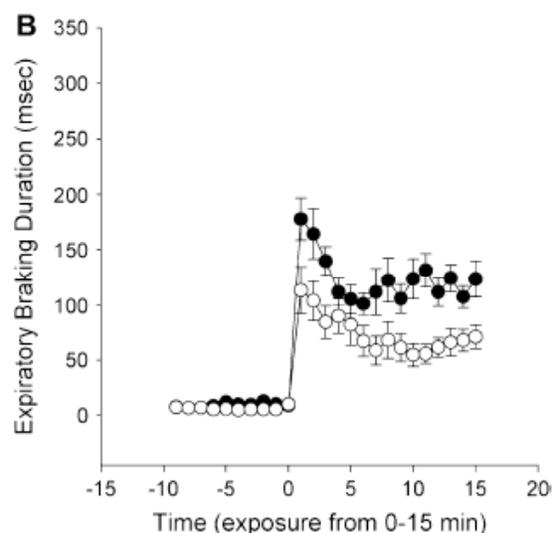


**FIGURE 3:** Shown are the average expiratory braking durations during each minute of baseline (minutes  $-9$  to  $0$ ) and aerosol exposure (minutes  $0$  to  $15$ ) of control (closed circles) and OVA-AAD mice (open circles) exposed to  $14$  mM adenosine aerosol. Repeated measures ANOVA revealed that expiratory braking durations were significantly higher than baseline levels during minutes  $1-4$  in control mice and throughout the entire exposure in OVA-AAD mice. The response was significantly higher in the OVA-AAD than in the control mice ( $p < 0.05$ , two-factor repeated measures ANOVA). Data are presented as mean  $\pm$  SEM; the groups contained  $8-10$  mice.

### *Adenosine Signaling: Contribution to Sensory Irritant Response to Irritant Vapors*

The effect of theophylline pretreatment on the sensory irritation response to acetic

acid is shown in Figure 4. Exposure groups contained three to five mice. Acetic acid exposure concentration averaged 110 ppm. Expiratory braking was increased over baseline by acetic acid throughout the entire exposure in both the control and theophylline-pretreated mice. The peak response of  $\sim 200$  ms occurred at the onset of exposure; at this time breathing frequency average 61% of the baseline frequency. This response was also greater than that observed for ATP or adenosine. Theophylline reduced the response to acetic acid. Two-factor ANOVA revealed a significant effect of pretreatment ( $p < 0.05$ ) and an effect of exposure time ( $p < 0.001$ ) but did not detect a significant interaction between these factors. Baseline breathing frequency was significantly higher in theophylline-pretreated animals averaging  $311 \pm 21$  in this group compared with  $267 \pm 12$  breaths per minute (mean  $\pm$  SD) in the control mice ( $p < 0.05$ ). Baseline minute ventilation was slightly higher in the theophylline group than in the control group averaging  $76 \pm 17$  and  $67 \pm 10$  ml/min (mean  $\pm$  SD), respectively, but these values did not differ significantly from each other ( $p > 0.05$ ).

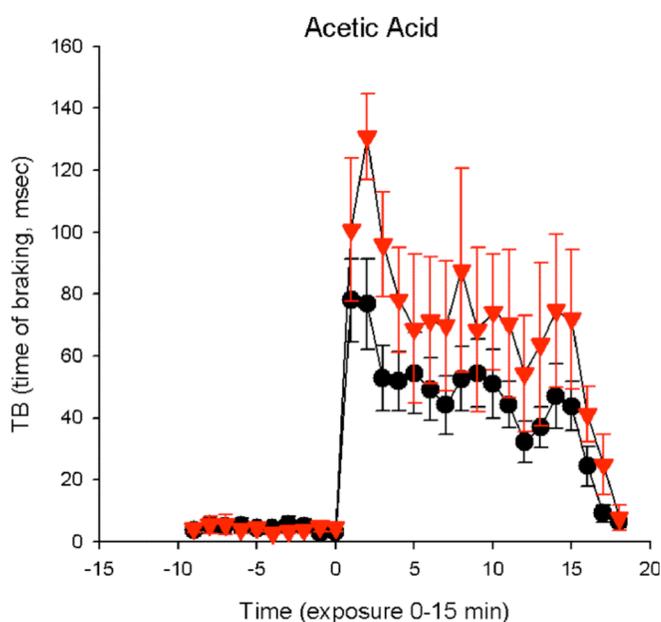


**FIGURE 4:** Shown are the average expiratory braking durations during each minute of baseline (minutes  $-9$  to  $0$ ) and acetic acid vapor exposure (minutes  $0$  to  $15$ ) of control (closed circles) and theophylline-pretreated (open circles) mice. Data are presented as mean  $\pm$  SEM. Groups contained 3-5 mice. In mice exposed to 110 ppm acetic acid, repeated measures ANOVA revealed that expiratory braking durations were significantly higher than baseline levels in both the control and theophylline groups. Two-factor ANOVA revealed a significant

effect of pretreatment ( $p < 0.05$ ), an effect of time ( $p < 0.05$ ), and no statistical interaction between these factors.

### *The Effect of OVA-AAD on the Response to Inspired Acetic Acid*

Shown in Figure 5 is the time of braking in response to inhaled acetic acid at 75 ppm of control and OVA-d8 mice. Exposure groups contained 6-11 mice. Two-factor ANOVA revealed a significant effect of OVA treatment ( $p < 0.01$ ), and a significant effect of time ( $p < 0.001$ ).



**FIGURE 5:** Shown are the time of braking durations during each minute of baseline (minutes  $-9$  to  $0$ ) and acetic acid aerosol exposure (minutes  $0$  to  $15$ ) in control (closed circles) and OVA-AAD day 8 (upside-down triangles) mice.

## Discussion

The sensory irritation response, a decreased breathing frequency due to a prolonged braking at the onset of the expiratory phase of each breath, is a specific response that has

long been known to result from trigeminal sensory nerve activation (Alarie 1973). Although the trigeminal nerves consist of both A $\delta$  fibers and C fibers, the role of each type in the sensory irritant response is not known. Previous studies showed that both might participate (Morris 2003, Symanowicz 2004). Previously our lab showed that ATP aerosols produce concentration-dependent sensory irritation responses in the mouse, and that this was receptor-mediated due to the effect of theophylline on the response. A similar response to adenosine confirmed that purinergic sensory transduction pathways exist for nasal trigeminal sensory nerve activation (Vaughan *et al* 2006). Rather than two separate pathways, ATP may act, in part, by being degraded to adenosine, as previously described.

In the current study, adenosine aerosol produced a sensory irritation response in control mice, statistically similar to that in capsaicin-pretreated mice (Figure 2). This suggests a significant role for capsaicin-insensitive A $\delta$  fibers compared to capsaicin-sensitive C fibers in the response to adenosine. This was already suspected, given the distribution of A receptors on A $\delta$  fibers rather than C fibers, and has been shown in other studies (Kollarik *et al* 2003). The A1-selective antagonist DPCPX significantly reduced the response to inhaled adenosine, suggesting a dominant role for the A1 receptor in the response. However, because it did not completely abolish the response, the response to adenosine may not be limited to A1 receptor activation. There are, of course, A2 and A3 receptors, and subtypes therein. In addition, adenosine may modify the response of C fibers to other stimulants, rather than stimulating the C fibers directly.

The sensory irritation response to adenosine aerosol challenge was enhanced in the OVA-allergic airway disease model (Figure 3). The mechanism, or more likely mechanisms, of the increase are not known. Higher inspired dose of adenosine is unlikely to be the reason,

because OVA-AAD mice had reduced minute ventilation and therefore received a lower total dose.

While the above response to adenosine is somewhat expected, there is much less understanding of how respiratory irritants like acetic acid cause nasal trigeminal sensory nerve activation. To address this, animals were exposed to acetic acid with and without capsaicin pretreatment. Compared to control mice, capsaicin pretreatment reduced the average time of braking by 34% across all concentrations of acetic acid (28, 43, and 84 ppm, data not shown). The effect was similar at all exposure concentrations, suggesting that the sensitivity of both A $\delta$  and C fibers to acetic acid was similar. Certainly if the thresholds for detection of acetic acid were much lower in C fibers, this would be reflected in differential effects of capsaicin pretreatment at lower adenosine exposure concentrations. This was not seen.

Because capsaicin pretreatment did not entirely abolish the response to acetic acid, further experiments were needed to discover other mechanisms that may be participating. Acetic acid was given with and without the non-selective A receptor antagonist theophylline (Figure 4). Theophylline reduced the braking duration by about half, and this effect was sustained throughout the active exposure period. The response to acetic acid is therefore also mediated through A receptors, presumably on capsaicin-insensitive nerve fibers.

Furthermore, the sensory irritation response to inhaled acetic acid in the OVA-AAD model was also enhanced compared to in healthy animals (Figure 5). This correlates well with the observation that human subjects with allergic rhinitis demonstrate enhanced responsiveness to acetic acid vapor (Shusterman *et al* 2005). In theory, adenosine blockade may be useful therapeutically in these patients.

There are several limitations to this study. It is possible that these chemicals are acting by other, non-specific mechanisms. For example, theophylline not only non-specifically antagonizes A receptors, but also likely inhibits phosphodiesterase (PDE) enzymes, may inhibit tumor growth factor beta (TGF- $\beta$ ), and may activate the enzyme histone deacetylase 2 (HDAC2). The systemic delivery of theophylline did affect baseline respiratory rate in some of the trials, but usually not enough to likely affect results. Regardless, inhaled theophylline might be superior. Acetic acid may be altering the pH of the intracellular environment, and acid-sensing ion channels (ASIC) may contribute to sensory neuron activation. DPCPX is a much less well characterized molecule, but is not above suspicion for having effects in addition to A1 receptor antagonism. DPCPX was administered systemically as well, after failure to find a suitable solvent for aerosol generation. The systemic administration of capsaicin may have additional unknown effects as well.

Another possible limitation is that mice do not have the exact same anatomy as humans. Mice are generally used as a respiratory model because they can be used manageably in these studies and are an acceptable model of the human respiratory system. There certainly is a risk, however, that there may be key differences between their bodies and ours. Even if mice do represent human respiratory systems perfectly, the role of adenosine as a paracrine signal in the response to inhaled irritants may not be a dominant mechanism. Chronic activation of this system may lead to changes in its relative importance in the body via down-regulation.

Future research may include exposing capsaicin-pretreated mice to acetic acid with and without theophylline or DPCPX. The complement to this would be OVA-AAD mice with and without theophylline, DPCPX, or capsaicin pretreatment. These would help show

the role of A receptors in the activation of A $\delta$  fibers. It would also allow comparison of the dominant mechanisms of sensory neuron activation in healthy compared to AAD subjects. Ultimately, the benefit in determining more specifically the mechanisms of these irritants is more narrowly targeted therapy. Targeting a particular receptor will help reduce unrelated side effects of medication, which tends to improve patient compliance, and the practical usefulness of a treatment.

In summary, adenosine appears to minimally affect C fibers in healthy animals, and to act predominately through A $\delta$  fibers via the A1 receptors. The response to acetic acid, however, appears to be mediated through both A $\delta$  fibers and C fibers in healthy animals. The moderate effect of C fiber destruction on the response to acetic acid shows that C fibers are involved. The role A $\delta$  fibers is revealed by the residual responsiveness in capsaicin pretreated mice as well as the effects of theophylline which highlights the role of A receptors, which are mostly localized to A $\delta$  fibers. The enhanced responsiveness to both adenosine and acetic acid in the OVA-AAD model suggests that the augmented adenosine signaling pathways may contribute to the enhanced responsiveness to acetic acid in the OVA-AAD model. If so, adenosine antagonist therapies may offer a novel therapeutic strategy for treatment of asthma, particularly for those individuals living in polluted environments.

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