Relaxant effect of superimposed length oscillation on sensitized airway smooth muscle

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Jo-Avila M, Al-Jumaily AM, Lu J. Relaxant effect of superimposed length oscillation on sensitized airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 308: L479–L484, 2015. First published December 5, 2014; doi:10.1152/ajplung.00218.2014.—Asthma is associated with reductions in the airway lumen and breathing difficulties that are attributed to airway smooth muscles (ASM) hyperconstriction. Pharmaceutical bronchodilators such as salbutamol and isoproterenol are normally used to alleviate this constriction. Deep inspirations and tidal oscillations (TO) have also been reported to relax ASM in healthy airways with less response in asthmatics. Little information is available on the effect of other forms of oscillations on asthmatic airways. This study investigates the effect of length oscillations (LO), with amplitude 1 and 1.5% in the frequency range 5–20 Hz superimposed on breathing equivalent LO, on contracted ASM dissected from sensitized mice. These mice are believed to show some symptoms such as airway hyperreactivity similar to those associated with asthma in humans. In the frequency range used in this work, this study shows an increase in ASM relaxation of an average of 10% for 1.5% amplitude when compared with TO, ISO, or the combination of both. No similar finding is observed with 1% amplitude. This suggests that superimposed length oscillation acting over the interaction of myosin and actin during contraction may lead to temporal rearrangement and disturbance of the cross-bridge process in asthmatic airways. No similar findings were observed with 1% amplitude. Deep inspirations and tidal oscillations (DI) have not been tested on asthmatic airways as a therapeutic alternative. Thus our interest focuses on testing the effect of different amplitudes and frequencies of SILO on contracted ASM from sensitized mice. It is expected that this would help to fill the knowledge gap and will allow us to learn more about the behavior of sensitized airways when SILO are applied during breathing.

Asthma is primarily characterized by reversible airway hyperconstriction, hyperresponsiveness, and inflammation. It is believed that the key effector of airway bronchoconstriction in asthma is attributed to airway smooth muscle (ASM) contraction (14). This contraction is normally relieved by using a pharmaceutical relaxant such as isoproterenol (ISO). Unfortunately, some relaxants are often associated with various degrees of side effects (7, 25). Searching for alternative treatments with less-harmful side effects has been the main objective of many investigators in this field of study (1, 3, 8, 9, 11, 12, 17, 20, 26, 29). At the cellular level, ASM contraction and relaxation are determined by the extent of the actin-myosin cross-bridge cycling process. With the activation of ASM, this biophysical process may be initiated by hormonal or neural stimuli followed by biochemical activities that result in relatively restricted movement between the myosin and actin filaments to produce muscle contraction. Pharmacological treatments are normally used to alleviate this contraction by either relaxing the constricted airways or reducing the inflammation observed. Alternatively, several in vitro studies have demonstrated that length oscillations (LO) equivalent to those occurring during tidal breathing or with some superimposed length oscillation (SILO) do disturb the cross-bridge cyclic process and produce some relaxation in precontracted ASM obtained from healthy airways (11, 12, 14, 32). Furthermore, it has been demonstrated that combining LO with ISO enhances the relaxation for contracted tissues obtained from healthy airways (17); particularly, it was indicated that ISO of 10^{-7} to 10^{-5} M caused the same force inhibition as tidal oscillations (TO). Examining the ISO dose-response curve and that of combined ISO with TO shows that the dose effect was almost superposed for all ISO concentrations except the highest one of 10^{-5} M. This suggests that the relaxation effects of ISO and TO are multiplicative and largely independent, i.e., deactivation vs. disruption of the myosin cross-bridge (17).

The above-cited studies were conducted on tissues isolated from healthy airways. However, recent studies such as Chin et al. (10) and Noble et al. (28) have focused on asthmatic airways, with the latter focusing on deep inspirations (DI) and TO effect. To the best of our knowledge, other LO different from those occurring during breathing and DI have not been tested on asthmatic airways as a therapeutic alternative. Thus our interest focuses on testing the effect of different amplitudes and frequencies of SILO on contracted ASM from sensitized mice. It is expected that this would help to fill the knowledge gap and will allow us to learn more about the behavior of sensitized airways when SILO are applied during breathing.

In spite of the fact that no animal model can fully resemble human asthma, it has been observed that some features like inflammation, airway hyperresponsiveness, and remodeling of the airways can be reproduced in animal models (16, 22–24). Mouse model presents advantages for physiological studies of asthma because of their size, short cycles of breeding, and existing data regarding sensitized models resembling various features of asthma. The recent advances in transgenic technology and the development of species-specific probes have allowed detailed mechanistic studies to be conducted on mouse models (4, 5, 33). The main purpose of using mice in this study was to generate an acute sensitized model capable of resembling closely airway hyperreactivity (AH) observed during an asthma attack. This will be significant in understanding how airways from healthy and sensitized mice respond in vitro to relaxant effect of superimposed length oscillation (SILO) do disturb the cross-bridge cyclic process and produce some relaxation in precontracted ASM obtained from healthy airways (11, 12, 14, 32). Furthermore, it has been demonstrated that combining LO with ISO enhances the relaxation for contracted tissues obtained from healthy airways (17); particularly, it was indicated that ISO of 10^{-7} to 10^{-5} M caused the same force inhibition as tidal oscillations (TO). Examining the ISO dose-response curve and that of combined ISO with TO shows that the dose effect was almost superposed for all ISO concentrations except the highest one of 10^{-5} M. This suggests that the relaxation effects of ISO and TO are multiplicative and largely independent, i.e., deactivation vs. disruption of the myosin cross-bridge (17).

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MATERIALS AND METHODS

All experimental procedures were approved by the University of Auckland Animal Ethics Committee according to the Code of Ethical Conduct and performed in accordance with the Animal Welfare Act 1999 New Zealand.

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**Animal Model and Sensitization**

We designed and used an ovalbumin (OVA) sensitization model (acute model) for this study, following a protocol similar to that of Kumar et al. (24). The mouse strain used during the study was Balb/c, female, between 8 and 12 wk old. They were selected due to the fact that this strain is highly reactive in respiratory studies and hence commonly used for sensitization protocols (27). The model included two methods of administration of the allergen (Fig. 1). First, intraperitoneal injections of OVA at 10 μg diluted in 0.9% sodium chloride (saline) + 1 mg aluminum hydroxide mixture were administered to the animals on days 0, 7, and 14 to induce immunological response. Second, after 10 days of the last intraperitoneal injection, aerial nebulization of OVA at 5%, diluted in saline, was used to expose the airways to the allergen for 20 min on days 24, 28, and 32. After the last nebulization, the mice were allowed to rest for 24 h and were culled on day 33 to obtain the tissue (tracheal rings) for testing.

### Tissue Preparation and Experimental Apparatus

Tracheal rings 2 mm wide were obtained from healthy and sensitized mice and were mounted in a 5-ml computerized organ bath system (800A in vitro test system; Aurora Scientific, Ontario, Canada) as previously described (21). Each tracheal ring was suspended vertically to a servo-controlled lever arm (model 300C; Cambridge Technology, Aurora Scientific) using steel wire and nonpenetrating hooks. The bath was filled with physiological Krebs solution (composition in mM: 110 NaCl, 0.82 MgSO4, 1.2 KH2PO4, 3.39 KCl, 2.4 CaCl2, 25.7 NaHCO3, and 5.6 glucose) and bubbled with a gas mix of 95% O2-5% CO2 to maintain the pH at 7.4. A constant temperature of 37°C was maintained by using a surrounding water jacket for the bath. Force and length were controlled using a dual-mode system (37°C was maintained by using a surrounding water jacket for the bath. Force and length were controlled using a dual-mode system (model 300C; Cambridge Technology, Aurora Scientific) using steel wire and nonpenetrating hooks. The bath was filled with physiological Krebs solution (composition in mM: 110 NaCl, 0.82 MgSO4, 1.2 KH2PO4, 3.39 KCl, 2.4 CaCl2, 25.7 NaHCO3, and 5.6 glucose) and bubbled with a gas mix of 95% O2-5% CO2 to maintain the pH at 7.4. A constant temperature of 37°C was maintained by using a surrounding water jacket for the buffer. Force and length were controlled using a dual-mode system (Dual Mode Servomotor model 300B; Cambridge Technology, Aurora Scientific). Signal data were acquired and sent via a National Instruments DAQ board to a LabVIEW program (NI-DAQ7/LabVIEW6) that was developed for this purpose. Reference diameter (D0) was determined with repeated cycles of stretching, contraction (10^-4 M ACh), and rinsing until the maximum contractile force (the force during contraction, also termed passive force) was reached. Subsequently, the airway was allowed to stabilize at D0 for 30 min.

### Experimental Protocols

For each testing protocol, the tissue was first constricted with 10^-4 M ACh until a stabilized plateau was reached. Subsequently J) 10^-6 M ISO, 2) oscillations, or 3) 10^-6 M ISO + oscillations were applied to the tracheal ring (Fig. 2). The concentrations used for ACh and ISO were previously determined and selected using dose-response curves for each drug. Following this, each tracheal ring was rinsed repeatedly with Krebs solution and allowed to recover before a subsequent test.

Assuming that airway compliance is similar to total lung compliance, the ASM length oscillation can be derived from the cube root of the lung volume changes (13, 18). Values were calculated to be about 4% for normal breathing and about 25–30% for DI (19). The following length oscillations were tested: 1) breathing oscillations with amplitude/frequency of 4%/2.7 Hz (determined using mice breathing patterns as reference) and 2) SILO with amplitude/frequency of 1% and 1.5%/5, 10, 15, and 20 Hz. The combined ISO-oscillations protocol was performed with 10^-6 M ISO. All oscillation amplitudes were calculated as percentage of D0.

### Evaluation of the Model

The following procedures were used to assess the model. **AH plethysmography.** AH was measured and evaluated in spontaneously tracheotomized mice through plethysmography, using lung resistance (Rl = tracheal pressure/flow rate) as a reference value. The objective of this technique was to evaluate bronchoconstriction in the control and sensitized animal model.

**ELISA.** In asthmatic models, the levels of the antibody IgE are normally increased. To confirm that our model showed some asthmatic symptoms, the level of this antibody was tested using a direct sandwich enzyme-linked immunosorbent assay (ELISA) method in duplicate. Blood samples from mice hearts were obtained right after the experimental protocols and using coagulation and centrifugation; samples of serum were prepared from it and then stored at −80°C. Next, IgE levels were quantified using the Mouse OVA-IgE ELISA kit from mbioproducts (division of mbiosciences, Zürich, Switzerland) and compared between controls and sensitized.

**Bronchoalveolar lavage.** Bronchoalveolar lavage (BAL) was used to recover an airway sample to evaluate and compare the cellularity of the healthy and sensitized mice. This technique involves successive lavages of the airways with physiological solutions. After the experimental protocols, the lungs were cannulated in situ and washed with 1 ml of saline (0.9% NaCl) several times before collecting a representative sample of the airways. Slides were prepared from the BAL and stained with hematoxylin and eosin. White cells (WC) were counted and differentiated in a blinded fashion by counting 100 cells by light microscopy. The number of eosinophils was expressed as a percent of the total WC.

### RESULTS

**Evaluation of the Model**

Respiratory plethysmography was used to diagnose sensitized models (15). Table 1 shows Rl, ELISA, and BAL results for both tested groups, healthy and sensitized. It shows an increase in airway response to 10^-4 M ACh in sensitized mice compared with healthy ones (n = 6 in each group). Rl increased from 1.62 ± 0.14 to 2.28 ± 0.16 (SE) cmH2O·ml^-1·s^-1. This is a typical feature of AH associated with asthma. Other parameters such as the levels of anti-OVA IgE and analyses of cellularity in BAL were also analyzed and found to increase in the sensitized model compared with the healthy mice. ELISA showed a significant increase of IgE with a P < 0.05. The BAL showed increased presence of WC and red and epithelial cells in all slides from sensitized slides. In fact, the healthy slides showed poor presence or complete absence of the same cellularity. A significant increase in eosinophils was observed (21 ± 6% compared with 3 ± 1%).

**In Vitro Testing**

Figure 3 shows the percentage relaxation (%R) for various conditions. %R was calculated as the total reaction force b (see Fig. 2) observed 5 min after the application of ISO and/or
oscillations relative to the force observed before these agents, which corresponds to the plateau (a) [%R = (a− b)/a] × 100. The statistical analysis was performed considering a normal distribution of the data and using a paired t-test for each dose of treatment (%amplitude, Hz, and ISO). Figure 3 shows that combining ISO with oscillations increases relaxation in healthy airways (as previously observed) compared with either ISO or breathing oscillations alone (≈43 ± 8.3, 47 ± 9, and 57 ± 9.30% respectively). However, this effect is missing in sensitized airways. In fact, the effectiveness of ISO, breathing oscillations, and both agents combined significantly reduced in sensitized airways compared with the effect of the same agents in healthy airways (≈3 ± 1.85, 5 ± 1.3, and 6 ± 3.2% respectively).

Breathing, ISO, and Both Agents Combined

Figure 4 compares the effect of SILO of 1% amplitude and frequencies of 5, 10, 15, and 20 Hz with the effect of breathing oscillations and ISO alone as well as combined together in sensitized airways. ANOVA and Wilcoxon signed-rank test were also used in these analyses. Figure 4 shows various responses can be observed but without any statistical significance except a significant increase in relaxation is observed at 5 Hz (P value <0.05 through t-test).

Table 1. Sensitization results for $R_L$, ELISA (blood levels of IgE), and BAL (level of eosinophils)

<table>
<thead>
<tr>
<th></th>
<th>$R_L$, cmH$_2$O·ml$^{-1}$·s$^{-1}$</th>
<th>ELISA (IgE), ng/ml</th>
<th>BAL (eosinophiles), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized mice</td>
<td>2.28 ± 0.16</td>
<td>125.67 ± 26.74</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>Healthy mice</td>
<td>1.62 ± 0.14</td>
<td>6.11 ± 3.82</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 mice in each group. $R_L$, lung resistance; ELISA, enzyme-linked immunosorbent assay; BAL, bronchoalveolar lavage.
Figure 5, on the other hand, shows that SILO with an amplitude of 1.5% and at the four frequencies tested in this work significantly increases relaxation (ANOVA and t-test, P < 0.05). The increased relaxation is 16 ± 2.07, 18 ± 3.39, 20 ± 9.14, and 14 ± 8.39% for a frequency of 5, 10, 15, and 20 Hz, respectively.

DISCUSSION

Muscle contraction is normally associated with the cross-bridge cycling process. This process generates force that displaces the thin actin filament along the thick filament, generating shortening of the muscle that results in muscle contraction. The pharmacological approach to alleviate this contraction focuses on the chemical pathways of the contraction to induce relaxation and does not take into consideration the mechanical components involved in the cross-bridge cycling process. However, there has been some evidence that the latter process can be disturbed by LO to produce muscle relaxation (12–14, 18, 19, 30, 32). During breathing, the lungs contract and expand, which results in changes of the airway wall diameter. Consequently, the ASM is exposed to continuous length changes. Studies on ASM from healthy airways have found that load fluctuations imposed continuously on ASM due to tidal breathing and DI have a reduction effect on force and muscle stiffness caused by stretch or mechanical oscillation (12–14, 18, 19, 30, 32). It has been hypothesized that these oscillations have the ability to disrupt the cross-bridge cycle process (17). Our previous in vitro study on isolated ASM tissue from healthy airways (1) further confirmed the force reduction effect of TO and the effect of SILO. With the use of the immunofluorescence staining technique, the latter study confirmed the occurrence of cross-bridge cycling disturbance.

To date, the effect of ISO and breathing (both tidal and inspiratory oscillations) on isolated ASM reactivity has been focused on tissues from healthy airways with the exception of two recent studies by Chin et al. (10) and Noble et al. (28). The latter study showed a decrease in the relaxant effect of DI in asthmatic bronchus compared with healthy subjects, but with no statistical significance. Other research has demonstrated that, in most cases, LO has the tendency to enhance the relaxation obtained by using ISO alone in healthy airways (1, 18). To the best of our knowledge, this is the first study that tests the effect of applying ISO in combination with TO as well as SILO on ASM tissues from sensitized mice.

Our study shows that the combined effect of ISO with LO has a bronchorelaxant effect, which reinforces previous findings in ASM from healthy tissue (1, 13, 19, 30, 32). The bronchorelaxant effect of ISO as a result of interaction with the receptors present in ASM cell membranes is responsible for key smooth muscle responses to chemical signals and seems to increase when combined with mechanical oscillations similar to breathing.
to breathing in healthy airways. However, this effect was not observed in the same manner in sensitized airways when tested at 4%/2.7 Hz conditions. This study also shows a decrease of effectiveness of breathing, ISO, and the combination (Fig. 3). The reduction in the relaxant effect of breathing in asthmatic airways has been attributed to temporary airway changes or adaptation of the ASM (6, 19, 31, 32) during the development of the disease.

SILO with amplitude of 1.5% and frequencies in the range of 5–20 Hz on breathing oscillations alone increase the relaxation of the sensitized airways compared with oscillations equivalent to breathing alone, ISO alone, or both combined. However, SILO with an amplitude of 1% in the same range does not result in similar findings. At all the tested frequencies except 5 Hz, the 1% amplitude shows a reduction in relaxation of sensitized airways compared with mechanical oscillations equivalent to breathing alone. However, no specific trend of relaxation can be observed.

All in vitro findings from healthy mice seem to confirm that physiological oscillations (mechanically reproduced) similar to those occurring during breathing can disturb the mechanical components of contraction rather than affecting chemical pathways, but they are not capable of reproducing the same relaxation pattern in sensitized airways. This could be in part due to the adaptation of the ASM to the new conditions associated with the disease. However, if SILO onto breathing patterns induces relaxation in preconstricted sensitized airways (where tidal oscillations have failed as observed in our study) then the main effect of contraction remains the same, namely cross-bridge cycling, and this could be disrupted. These data, in addition to previous data (1), seem to indicate that the relaxation of ASM in healthy and sensitized airways is closely related to the cross-bridge rate (the speed of attachment of myosin and actin elements), and this rate could be disrupted by changes in amplitude and frequency independently. Unfortunately, the rate (speed) of attachment of myosin and actin is unknown and depends on the stimulation process. Thus, further investigations are needed to determine which frequency values are the best to induce relaxation on these types of airways. These findings also need to be tested and probed in vivo conditions to advance toward a possible new therapy to treat AH in asthmatic attacks.

The reasons of the difference in response observed with the two tested amplitudes (1 and 1.5%) are not completely clear. This is one of the first studies testing different superimposed oscillations over breathing, and it will require further investigation to identify the underlying mechanisms behind this. At this stage, we can only speculate that the different strain (and its magnitude) used could be responsible for this difference, as previously observed with direct TO or DI (no superimposed) on healthy and asthmatic airways (2, 10, 28), and that a higher amplitude would be more likely to induce relaxation (1.5% or higher) on asthmatic subjects than a small amplitude (1%), but more studies in the area would be required to clarify this.

In summary, oscillations similar to those occurring during breathing seem to be ineffective in inducing relaxation in asthmatic airways similar to that observed in healthy airways. However, some of the SILO tested in this study were capable of inducing relaxation on sensitized airways (which presented AH). The SILO results currently support the hypothesis that LO acting over the interaction of actin and myosin during contraction may lead to temporal rearrangement and disturbance of the cross-bridge process in asthmatic airways. More studies with LO different from those occurring during breathing are essential to determine if there are any side effects to the airways and which oscillation parameters are the most effective and safest to be used for future alternative drug-free asthma therapies.

DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES