

The gain of smooth muscle's contractile capacity induced by tone on in vivo airway responsiveness in mice

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Lee-Gosselin A, Gendron D, Blanchet MR, Marsolais D, Bossé Y. The gain of smooth muscle's contractile capacity induced by tone on in vivo airway responsiveness in mice. *J Appl Physiol* 118: 692–698, 2015. First published January 8, 2015; doi:10.1152/jappphysiol.00645.2014.—Airway hyperresponsiveness to a spasmogenic challenge such as methacholine, and an increased baseline tone measured by the reversibility of airway obstruction with a bronchodilator, are two common features of asthma. However, whether the increased tone influences the degree of airway responsiveness to a spasmogen is unclear. Herein, we hypothesized that increased tone augments airway responsiveness in vivo by increasing the contractile capacity of airway smooth muscle (ASM). Anesthetized, tracheotomized, paralyzed, and mechanically ventilated mice were either exposed (experimental group) or not (control group) to tone for 20 min, which was elicited by nebulizing serial small doses of methacholine. Respiratory system resistance was monitored during this period and the peak response to a large cumulative dose of methacholine was then measured at the end of 20 min to assess and compare the level of airway responsiveness between groups. To confirm direct ASM involvement, the contractile capacity of excised murine tracheas was measured with and without preexposure to tone elicited by either methacholine or a thromboxane A₂ mimetic (U46619). Distinct spasmogens were tested because the spasmogens liable for increased tone in asthma are likely to differ. The results indicate that preexposure to tone increases airway responsiveness in vivo by $126 \pm 37\%$ and increases the contractile capacity of excised tracheas ex vivo by $23 \pm 4\%$ for methacholine and $160 \pm 63\%$ for U46619. We conclude that an increased tone, regardless of whether it is elicited by a muscarinic agonist or a thromboxane A₂ mimetic, may contribute to airway hyperresponsiveness by increasing the contractile capacity of ASM.

asthma; airway hyperresponsiveness; airway smooth muscle; tone; spasmogen; contractile capacity

AIRWAY HYPERRESPONSIVENESS (AHR) to an inhaled spasmogen and an exaggerated response to an inhaled bronchodilator are two typical features of asthma, so much that they both became objective criteria for diagnosing asthma (33). The exaggerated response to a bronchodilator testifies that tone (which is defined herein as a sustained contractile activation of ASM) is elevated in asthma (32). It is also established that the extent of tone correlates with the degree of airway responsiveness both in healthy individuals (6) and in patients with asthma (5). However, whether a link of causality exists between the extent of tone and the degree of airway responsiveness has never been ascertained.

We recently demonstrated that tone elicited by the continuous presence of a spasmogen increases the contractile capacity of ovine tracheal strips within a time scale of minutes (10, 11, 36). This gain of ASM contractile capacity caused by tone is

relevant to the understanding of AHR. In fact, it supports tonic activation of ASM as an underlying contributor of AHR in asthma. Yet whether the gain in contractile capacity caused by an increased tone occurs in vivo is unknown. This matter represents the gist of the present study. We used mice to investigate whether an elevated tone induced by repetitive challenges with low doses of methacholine (MCh) alters the subsequent response to a challenge with a high dose of MCh. The results demonstrate that the degree of airway responsiveness in vivo is greatly enhanced by exposure to a preceded elevated tone. We then determined whether this result can be attributed to the contractile plasticity of ASM. The contractile capacity of excised murine tracheas was tested in the absence or presence of a preceded tone elicited by either MCh or another spasmogen; namely, the thromboxane A₂ mimetic U46619. Together, the results suggest that a gain in contractile capacity can be accountable for the increase in airway responsiveness observed in vivo after an elevated tone.

MATERIAL AND METHODS

Animals. Nine- to 14-wk-old male and female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used for both in vivo and ex vivo experiments. The protocols were approved by the Committee of Animal Care of Laval University in accordance with the guidelines of the Canadian Council on Animal Care.

In vivo airway responsiveness. In vivo airway responsiveness was assessed by measuring the changes in respiratory system resistance (Rrs) induced by intratracheal nebulization of MCh in live, anesthetized, tracheotomized, paralyzed, and mechanically ventilated mice. Specifically, mice anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively) were tracheotomized and connected to a computer-controlled ventilator (FlexiVent; SCIREQ, Montreal, QC, Canada) at a respiratory frequency of 150 breaths/min, a tidal volume of 10 ml/kg, and a positive end-expiratory pressure of 3 cmH₂O. Once ventilation was established, the mice were paralyzed with 0.1 mg/kg of pancuronium bromide injected intramuscularly. Rrs was measured by the FlexiVent using the perturbations dubbed the Snapshot-150. Heart rate was monitored continuously by electrocardiography throughout the experiment to ensure proper anesthesia.

The dosing regimen for MCh delivery is illustrated in Fig. 1. Age and sex-matched mice were either exposed (experimental group) or not (control group) to a tone for 20 min before being administered a final large dose of MCh. MCh was delivered by nebulization during tidal breathing, and deep inspiration was omitted throughout the entire protocol. Tone in the experimental group was induced with four small doses of MCh administered at 5-min intervals; an initial dose of 10 mg/ml followed by three doses of 5 mg/ml. A final dose of 75 mg/ml was then delivered 5 min after the last dose of 5 mg/ml. At each MCh dose, Rrs was measured three times before and 12 times after the administration of MCh at 9-s intervals. The same perturbations that were required to measure Rrs were performed in mice of the control group not exposed to tone. However, only one single dose of 100 mg/ml of MCh was delivered at the end in the control group. The purpose of the single 100 mg/ml dose was to equate the total quantity

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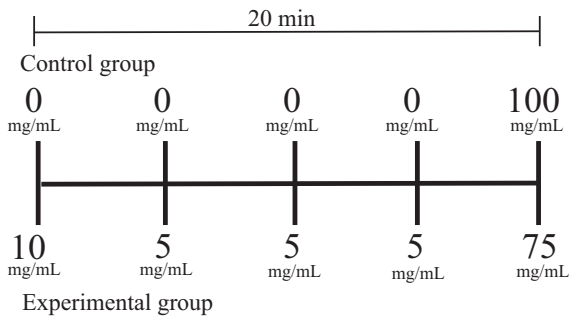


Fig. 1. Protocol for measuring whether exposure to an elevated tone increases airway responsiveness in mice in vivo. Mice were either exposed (experimental group) or not (control group) to an elevated tone induced by four small doses of methacholine (MCh) administered 5 min apart. A final large dose (75 mg/ml or 100 mg/ml) was then administered and the respiratory system resistance (Rrs) that was obtained was compared between groups of sex- and age-matched mice.

of MCh delivered in the experimental group [$100 \text{ mg/ml} = 10 + (3 \times 5) + 75 \text{ mg/ml}$]. Rrs in response to the final dose was then compared between the control and the experimental groups. In an additional set of experiments we demonstrated that the diluent alone (i.e., saline) neither induced tone nor affected the responsiveness to 100 mg/ml of MCh (data not shown).

Contractility of excised murine tracheas. Mice were euthanized with ketamine-xylazine (200 and 10 mg/kg, respectively) and the trachea was placed into Krebs solution (pH 7.4, 111.9 mM NaCl, 5.0 mM KCl, 1.0 mM KH_2PO_4 , 2.1 mM MgSO_4 , 29.8 mM NaHCO_3 , 11.5 mM glucose, and 2.9 mM CaCl_2). The whole trachea was then mounted in a 40-ml organ bath containing Krebs solution maintained at 37°C between two platinum electrodes (2 mm wide $\times$ 50 mm long). A distending force of 5 mN (the resting tension) was applied parallel to the axis of force transmission of the tracheal ASM bundles. The trachea was connected by a surgical thread to a dual-mode lever arm system (300C; Aurora Scientific, Aurora, ON, Canada), which allowed measurements of resting tension and isometric force deployed by ASM in response to electrical field stimulations (EFS) and spasmogens. Before the measurements were made, the trachea was subjected to a period of equilibration of 45 min, during which time ASM was stimulated to contract every 5 min for 15 s with EFS (60 Hz, 20 volts, 2 ms).

Following the equilibration period, a cumulative concentration-response curve was generated with either MCh from 10^{-8} to 10^{-4} M or the thromboxane A_2 analog (U46619) from 10^{-10} to 10^{-5} M in log increments. This was to determine the concentration that induced 30% of the maximal response (EC_{30}) in each trachea. The trachea was then subjected to two sequences in a randomized fashion, separated by a washout to reestablish the resting tension. Throughout the experiment, each trachea was exposed to only one spasmogen. For the control sequence, the trachea was unstimulated for a period of 30 min, after which it was stimulated with 10^{-4} M MCh, 10^{-5} M U46619, or 80 mM KCl. These concentrations were chosen because they trigger the maximal response that can be obtained with these spasmogens. The peak force obtained during the first 5 min of contraction was recorded and used as a surrogate for the contractile capacity of ASM. For the experimental sequence, the trachea was stimulated with the EC_{30} of MCh, the EC_{30} of U46619, or 20 mM KCl for a period of 30 min. Following this 30 min of tone, the Krebs solution containing the EC_{30} of MCh, the EC_{30} of U46619, or 20 mM KCl was changed for a Krebs solution containing 10^{-4} M MCh, 10^{-5} M U46619, or 80 mM KCl, respectively. The peak force obtained during the 5 min following this change of concentration was recorded and used as a surrogate for the contractile capacity of ASM. The response to 10^{-4} M MCh, 10^{-5} M U46619, or KCl 80 mM was compared between the two sequences to

determine whether preexposure to tone affects the contractile capacity of ASM.

At the end of the experimental sequence, the kinetics of reversal of the gain in force induced by tone was also measured. Specifically, MCh or U46619 was washed from the organ bath by changing the Krebs solution twice every 2 min until the recorded force had returned to resting tension. The resting tension was then maintained constant for 1 min prior to stimulation with either 10^{-4} M MCh or 10^{-5} M U46619. Peak force was recorded, and this entire procedure (washing and contraction) was repeated until the force generated by either MCh or U46619 was back to normal (i.e., to the force generated in response to 10^{-4} M MCh or to 10^{-5} M U46619 prior to exposure to tone).

An additional set of experiments was performed in which the duration of tone on the contractile capacity of ASM was investigated. The two sequences described above were thus repeated four times within the same tissues, each time with a different duration of tone exposure. Specifically, the contractile capacity of ASM in response to MCh at 10^{-4} M was assessed at 5, 10, 20, and 30 min following the introduction of tone elicited by the EC_{30} of MCh.

Statistical analyses. Data are shown as means $\pm$ SE. An unpaired *t*-test was used to compare maximal Rrs obtained after the administration of the final dose of MCh between the control and the experimental groups. Paired *t*-tests were used to compare the contractile capacity of ASM between sequences with and without preceded tone in response to either MCh (10^{-4} M) or U46619 (10^{-5} M). A repeated-measures two-way ANOVA was used to assess the kinetics of the gain of force induced by tone. Fisher's least significant difference (LSD) tests were then used to determine from which sequence duration that tone significantly alters the contractile capacity of ASM. Repeated-measures one-way ANOVAs with Fisher's LSD tests were used to assess the kinetics of return-to-normal contractile capacity after abatement of tone by spasmogen removal. All statistical analyses and graphs were performed using GraphPad Prism (San Diego, CA). $P < 0.05$ was considered sufficient to reject the null hypothesis.

RESULTS

In vivo airway reactivity. To assess whether an increased tone in vivo influences the degree of airway responsiveness in mice, the change in Rrs in response to MCh was measured following a prolonged exposure to tone and compared with a control group that was not exposed to tone. Figure 2 demonstrates Rrs measured throughout the protocols in each group. In the control group, Rrs remained constant until the final single dose was administered. In the experimental group, the response to every dose can be readily observed. Interestingly, the response to 5 mg/ml (doses 2, 3, and 4) increased over time. Rrs prior to each MCh dose in the experimental group progressively increased over time compared with that in the control group. This confirms that the dose regimen was effective in maintaining a greater tone between doses. Following delivery of the final dose, Rrs was $3.9 \pm 0.5 \text{ cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$ in mice in the control group and $8.9 \pm 1.7 \text{ cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$ in mice exposed to tone ($P = 0.01$). This demonstrates that airway responsiveness to the final MCh challenge was enhanced by $126.2 \pm 36.6\%$ in mice preexposed to an increased tone compared with the control group. Importantly, this cannot be due to a cumulative effect of MCh in the experimental group because the same total amount of MCh was delivered in both groups. In fact, the final dose in the experimental group (75 mg/ml) was lower than the final dose in the control group (100 mg/ml). The results were also segregated according to sex (data not shown). Although the effect seemed to be greater in

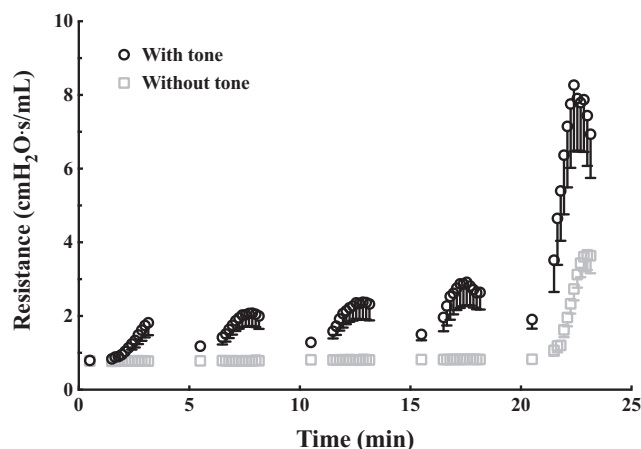


Fig. 2. An elevated tone increased airway responsiveness in vivo in mice. In the experimental group (circles), tone was induced for 20 min by delivering MCh at 5-min intervals. Rrs was measured three times before (average is shown), and 12 times (at 9-s intervals) after each dose. In the control group (squares), the same perturbations were performed, except that MCh was not delivered until the final dose. The final doses were adjusted so that the total quantity of MCh delivered in both groups was the same, as shown in Fig. 1. The total (cumulative) dose was 100 mg/ml in each group. The gain in Rrs induced by the final dose of MCh was $126.2 \pm 36.6\%$ greater in the experimental group compared with the control group ($P < 0.05$, $n = 10$ (20 mice)).

males, the phenomenon occurred in both sexes. Specifically, the gain in Rrs induced by the final dose of MCh was $173.8 \pm 67.5\%$ and $78.6 \pm 18.5\%$ greater in the experimental group compared with the control group in males and females, respectively ($P < 0.05$).

Contractility of excised murine tracheas. The experiments performed ex vivo were designed to assess whether a prolonged exposure to tone affects the contractile capacity of ASM. Figure 3 demonstrates the force generated by excised murine tracheas in response to MCh (Fig. 3A), U46619 (Fig. 3B), or KCl (Fig. 3C) throughout the two sequences explained in MATERIALS AND METHODS. Whereas the tracheas were kept unstimulated for 30 min in the control sequence, tone was introduced in the experimental sequence by adding the EC₃₀ of

MCh (Fig. 3A), the EC₃₀ of U46619 (Fig. 3B), or 20 mM KCl (Fig. 3C). Depending on the trachea, the EC₃₀ ranged from 3 to 8×10^{-7} M for MCh and from 1 to 5×10^{-8} M for U46619. Because the spasmogen was not cleared over time within the organ bath, repetitive administration was not required to maintain tone for 30 min in the ex vivo experiments. The contractile capacity of the tracheas was then assessed in response to a large concentration of MCh (10^{-4} M) (Fig. 3A), U46619 (10^{-5} M) (Fig. 3B), or KCl (80 mM) (Fig. 3C). As can be seen in Fig. 3, A and B, the force generated by these later concentrations was greater following preexposure to tone. In fact, the response was $22.5 \pm 4.4\%$ greater for tone elicited by MCh ($P = 0.005$) and $159.7 \pm 63.1\%$ greater for tone elicited by U46619 ($P = 0.017$). In contrast, tone induced by KCl was not able to increase the contractile capacity of murine tracheas in response to 80 mM KCl ($P = 0.72$). Similar experiments were performed in which the time of exposure to tone elicited by MCh varied from 5 to 30 min. The results are presented in Fig. 4, where it is demonstrated that the gain of force induced by tone requires time (note that the vertical distance between the gray and black symbols progressively increases over time). On the basis of a repeated-measures two-way ANOVA, the effect of tone was significant ($P = 0.01$). Tests a posteriori demonstrated that the enhancement of contractility induced by tone was significant at 20 ($P = 0.04$) and 30 min ($P = 0.02$), but not at 5 ($P = 0.47$) and 10 min ($P = 0.28$).

To assess the kinetics by which the gain of force induced by tone was reversed, tone was abated by spasmogen removal and the contractile capacity of ASM was retested at different time intervals. Results are presented in Fig. 5A for MCh and in Fig. 5B for U46619. It can be seen that after abatement of tone by spasmogen removal, the contractile capacity of the tracheas in response to a large concentration of spasmogen (10^{-4} M MCh or 10^{-5} M U46619) returned to the level obtained prior to tone exposure within 6.2 min for MCh and 15.0 min for U46619.

DISCUSSION

The results of this study indicate that an increased tone evoked by serial doses of a spasmogen increases airway responsiveness in vivo in mice. Ex vivo experiments performed

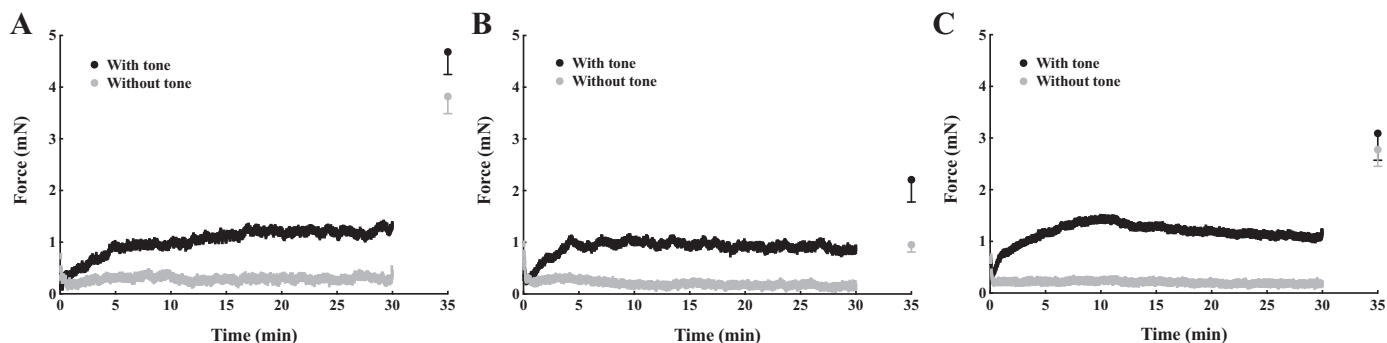


Fig. 3. A tone increased the absolute contractile capacity of excised murine tracheas regardless of the spasmogen used to elicit tone. A: tracheas were left unstimulated (gray) or exposed to tone elicited by the EC₃₀ of MCh (black) for 30 min. The gray and black traces are the means of all the experiments. The tone was induced by concentrations ranging from 3 to 8×10^{-7} M. The force-generating capacity was then measured in response to 10^{-4} M MCh. The response was $22.5 \pm 4.4\%$ greater in the sequence preceded by tone compared with the sequence without tone ($P < 0.05$). B: tone was induced by concentrations of U46619 ranging from 1 to 5×10^{-8} M. The force-generating capacity was then measured in response to 10^{-5} M U46619. The response was $159.7 \pm 63.1\%$ greater in the sequence preceded by tone compared with the sequence without tone ($P < 0.05$). C: tone was induced by 20 mM KCl. The force-generating capacity was then measured in response to 80 mM KCl. The response was not different between the sequences with and without preceded tone; $n = 5$ (A), $n = 7$ (B), and $n = 9$ (C).

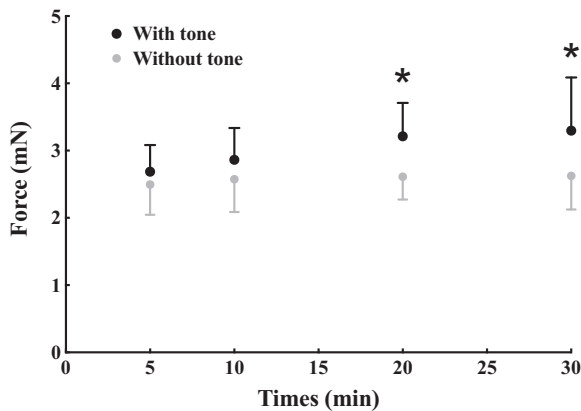


Fig. 4. The kinetics of the gain in force induced by tone. The contractile capacity of murine tracheal rings was measured in response to a concentration of 10^{-4} M MCh with and without preexposure to a tone induced by the EC₃₀ of MCh. Tone was induced for a period of 5, 10, 20, and 30 min. The response was 7.7, 11.3, 23.0, and 25.6% greater in the sequences preceded by 5, 10, 20, and 30 min of tone, respectively, compared with the corresponding sequences of same duration without tone. *Comparisons were statistically significant starting at 20 min (at 20 min $P = 0.04$; at 30 min $P = 0.02$). Tone was induced by concentrations ranging from 6 to 9×10^{-7} M; $n = 5$.

in murine tracheas further demonstrate that tone increases the contractile capacity of ASM. This gain in contractile capacity induced by tone occurred regardless of whether tone was elicited by MCh or a thromboxane A₂ mimetic, and in both cases it was reversible upon abatement of tone by spasmogen removal. Together, these results demonstrate that tone augments airway responsiveness by increasing the contractile capacity of ASM. It also lends additional support to the possibility that the increased tone observed in individuals with asthma is a major contributor to AHR.

New findings in relation to previous results. The present work represents important incremental work from three previous studies performed in an organ bath using ovine tracheal smooth muscle (10, 11, 36). In the first two studies we described a phenomenon by which the continuous exposure to a contractile agonist (acetylcholine; ACh) not only triggers a sustained contraction (i.e., tone) of ASM, but also increases its electrically triggered contractile capacity over time (10, 11). In the subsequent study, we demonstrated in the same ex vivo setup that this gain in contractile capacity occurs in oscillating conditions that simulate breathing maneuvers (36). Together, these findings reinforced the possibility that this phenomenon may be operational in vivo and may thus contribute to AHR, especially in individuals who present an increased tone, such as those with asthma (32). In the present manuscript we demonstrate for the first time that the gain in ASM contractility induced by an increased tone occurs in vivo. We also confirm that this is due to an increased contractile capacity of ASM by studying isolated murine tracheas in an organ bath with or without preceded tone elicited by the continuous presence of the contractile agonist MCh. We also demonstrate for the first time that the gain of contractile capacity occurs independently of the G protein-coupled receptor (GPCR) agonist used to elicit tone. Indeed, a sustained activation of ASM with the thromboxane A₂ mimetic U46619 was even more effective than MCh in increasing the contractile capacity of isolated murine tracheas. Owing to the molecular heterogeneity of asthma

pathogenesis (21, 40), this later finding is highly relevant. This is because the increased tone observed in asthma is likely mediated by diverse spasmogens. In fact, it is probably more realistic to postulate that a number of various spasmogens conspire in any given individual with asthma to increase tone. In all previous studies that have investigated the effect of tone on the contractile capacity of ASM ex vivo, the elevated tone observed in asthma was modeled by using a muscarinic agonist (ACh, MCh, or carbachol) (10, 11, 36). It was therefore important to determine whether other spasmogens have the ability to increase the contractile capacity of ASM, especially inflammation-derived spasmogens that are overexpressed in the airways of individuals with asthma. In this study we tested the effect of tone elicited by U46619. U46619 is a stable analog of thromboxane A₂, an inflammatory spasmogen that is upregulated after an allergen challenge in those with asthma (39, 41). U46619 induces ASM contraction via a paracrine mode of action whereby it triggers the release of ACh from the nerve endings, which then binds on the M₃ receptor on ASM to elicit contraction (17, 37), albeit not in canine bronchial ASM (23). On the basis of this documented mode of action of U46619 on ASM contractility, we cannot yet exclude the

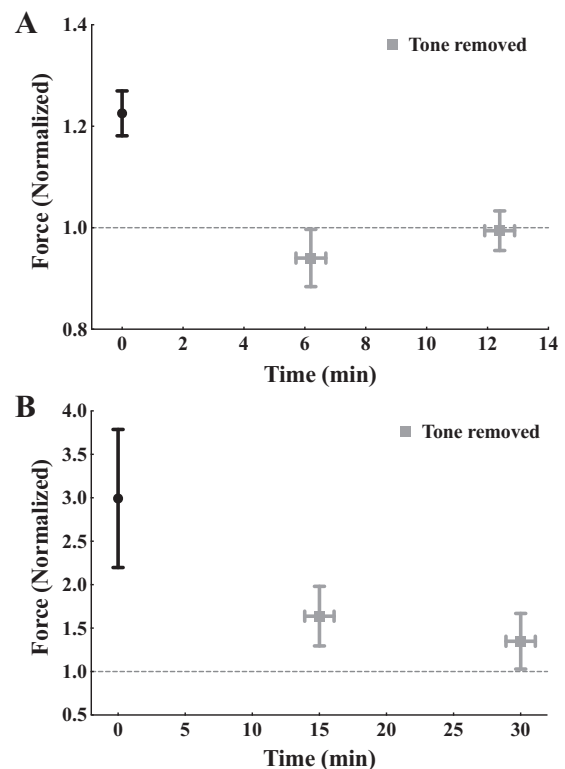


Fig. 5. The reversibility of the gain in force induced by tone elicited by either MCh (A) or U46619 (B). Force on the vertical axes was normalized to the force obtained in response to 10^{-4} M MCh or 10^{-5} M U46619 in the control sequence. A: the black circle shows the normalized force generated by tracheal airway smooth muscle at 10^{-4} M MCh at the end of the experimental sequence (following 30 min exposure to tone). Tone was then abated by washing out the spasmogen and the contractile capacity was retested two subsequent times (gray squares) in response to 10^{-4} M MCh once the tone had vanished (i.e., when the force generated by the EC₃₀ of MCh had vanished and resting tension was reestablished for at least 1 min). B: the same experiments were performed in tracheas exposed to U46619. The time required to eliminate the tone induced by MCh and U46619 varied between tracheas; $n = 5$ in both A and B.

possibility that the gain in force induced by tone is restricted to cholinergic stimulation. However, that an analog of a spasmogen that is upregulated in asthma enhanced the contractile capacity of ASM within a time scale of minutes bolsters the possibility that the phenomenon we reported may be operational in vivo and therefore play a role in asthmatic AHR.

However, the reason tone elicited by U46619 was more potent than that elicited by MCh is not clear. It might be important to mention that although U46619 induces contraction through the release of ACh (17, 37), the thromboxane receptor (TP) is expressed on ASM (17). Consequently, we suspect that both TP and M₃ receptors were activated during exposure to tone elicited by U46619. The simultaneous binding of two GPCRs is bothersome because the combined contractile effect of GPCR agonists can be synergistic (18, 19). Indeed, U46619 was previously shown to synergize with muscarinic agonists in vivo in humans (25), mice (17), and guinea pigs (38), but not in dogs (24). Synergistic effects between U46619 and muscarinic agonists were also observed in excised lung and precision-cut lung slices of mice (20), but not in bovine tracheal strips (15). The effect of thromboxane A₂ on ASM contraction is thus intricate. It depends on the species studied and on the current and past inflammatory state of the lung (17). In noninflamed airways of mice, such as in the present study, the TP receptor specifically expressed on ASM is responsible for the sensitization to MCh (17). To avoid synergistic effects between GPCR agonists, we formerly used a single spasmogen to induce tone and to assess the contractile capacity of ASM (10, 11, 36). However, now that we are aiming to build upon the physiological significance of this phenomenon by using spasmogens that are overexpressed in asthma to induce tone, we are no longer controlling for this potential confounder. Therefore, we suggest that the synergistic effect between the TP and the M₃ receptors accounted for the greater ability of U46619 than MCh to increase the contractile capacity of ASM.

Surprisingly, tone elicited by KCl was not able to enhance the contractile capacity of ASM. This result suggests that tone induced by the simple entry of Ca²⁺ from the extracellular compartment, or the simple mechanical effect of tone, is not sufficient to enhance the contractile capacity of ASM. Other intracellular pathways activated by GPCR, perhaps independent of the cytoplasmic increase in Ca²⁺ concentration, may be required for ASM to acquire additional force in response to tone. Many intracellular mechanisms can come into play to explain the gain in force induced by tone. Based on the current state of knowledge, the following are the most likely: 1) myosin (26) and actin (43, 46, 47) filamentogenesis; and 2) improved efficiency of the mechanotransduction by reinforcement of the adhesomes (22, 35, 43–47), which are multiprotein modules that bind to the cytoplasmic tail of integrins and that are responsible for linking the forces generated by the contractile apparatus inside the cells to the extracellular matrix.

Rapid change in ASM contractile capacity. The kinetics of the gain in contractile capacity induced by tone and its reversibility are fast (i.e., within a time frame of minutes). This raises serious questions regarding its physiological significance to asthmatic AHR. However, it is important to recognize that the origin of AHR is manifold (2). The factors that contribute to AHR can be either inherent or acquired due to inflammatory processes that take place in asthmatic lungs. In turn, the acquired defects can be either transient or more permanent

(13). The transient defects are generally the ones responsive to treatments and are responsible for the variable component of AHR. Among others they include edema, inflammatory infiltrates, neural reflexes, and mucus hypersecretion. The permanent defects are more refractory to current asthma therapies and are responsible for part of the fixed (or hardly modifiable) component of AHR. Among others, they include airway wall remodeling, loss of parenchymal recoil, and ASM enlargement. Owing to the kinetics of the gain in force induced by tone and its quick reversibility upon abatement of tone by spasmogen removal, we believe that the phenomenon we reported herein contributes to the acquired but transient component of AHR.

This quick change in ASM contractile capacity is susceptible to influence the level of airway responsiveness in many circumstances. The measurement of airway responsiveness is customarily assessed by administering serial escalating doses of a spasmogen (usually MCh). This takes several minutes to perform, which is beyond the time needed for the gain in force induced by tone to take place. Thus the quick gain in ASM contractile capacity that occurs in response to tone emphasizes the importance of designing experimental protocols that properly control for the time taken to administer serial doses of the spasmogen. In that regard, our results provide a likely explanation to earlier findings showing that an abbreviated method to deliver MCh doses leads to a significantly lower level of airway responsiveness in humans (34). Indeed, and despite the delivery of a higher cumulative dose and the same frequency of deep inflations [the latter is important to control because the time intervals between doses can seriously affect the degree of airway responsiveness (29)], the abbreviated methods resulted in a lower degree of airway responsiveness (34). This early finding was certainly counterintuitive because one would expect that the actual cumulative dose (i.e., the one remaining locally into the airways to act on ASM) to be greater during a fast delivery of MCh. This is because MCh inevitably wears off over time due to both degradation and washout by circulating blood (4, 14, 31). We propose that the decreased responsiveness may have been due to the contractile plasticity of ASM, which was heretofore ignored. In other words, the shorter time during which ASM was exposed to MCh may have attenuated the gain in contractile capacity induced by tone and thereby be accountable for the decreased responsiveness observed with the abbreviated method. In combination, these results also suggest that the magnitude of the gain in force induced by tone is not small. The lower level of airway responsiveness obtained with an abbreviated method of MCh delivery (34) implies that the rate of the gain in contractile capacity induced by tone is greater than the rate of decline in contractile activation caused by MCh clearance. More studies are warranted to clarify this premise.

The effect seems greater in vivo than ex vivo. For the same spasmogen, the gain in contractile capacity obtained ex vivo in tracheas was relatively smaller compared with the effect observed in vivo. Indeed, MCh increased airway responsiveness by 126% in vivo and increased ASM force by only 23% ex vivo. However, it is important to realize that the relationship between ASM force and respiratory system resistance is not linear. Previous computational analyses have suggested that a ~15% gain in ASM force can enhance airway resistance to airflow by up to 400% (9). Indeed, airway constriction is

greatly potentiated by geometric effects. Airway narrowing is related to ASM shortening at the second power [airway cross-sectional area = $\pi \times (\text{ASM perimeter}/2\pi)^2$], and airway resistance to airflow is inversely related to the radius of the airway lumen at the fourth power [airflow resistance = $8 \times \text{airway length} \times \text{air viscosity} \times (\pi \times \text{radius}^4)^{-1}$]. Apart from the nonlinear relationship between ASM force, ASM shortening, and luminal narrowing, it is also important to realize that many nonmuscle factors can influence the change in respiratory system resistance during an MCh challenge (12). Therefore, the reported gain in contractile capacity may lead to a functionally amplified response in vivo because it synergizes with nonmuscle factors to affect the change in Rrs induced by MCh. Together, the results highlight the important influence of small changes in the contractile capacity of ASM in affecting the degree of airway responsiveness in vivo in response to an inhaled spasmogen.

Limitations. Although our in vivo data represent solid evidence that tone enhances in vivo airway responsiveness by increasing the contractile capacity of ASM, we cannot exclude the confounding effect of airway heterogeneity. Indeed, airway heterogeneity can substantially influence Rrs (28). In our experiments, it is possible that an incremental number of small airways were closing over time during exposure to tone. In turn, this may have promoted airway heterogeneity and thereby amplified the gain in Rrs for any given average reduction in airway caliber elicited by the last dose of MCh. Consequently, the gain in Rrs induced by preceded tone in our in vivo study may be due to an amplified effect on airway heterogeneity rather than being purely due to an enhanced average narrowing of the airways caused by a greater force generated by ASM. Because our in vivo results are subject to different interpretations, we assessed the effect of tone on the contractile capacity of ASM at a smaller scale of biological length. Specifically, we assessed the effect of tone on the contractile capacity of excised tracheas. The results support the contention that the increased airway responsiveness observed in vivo following a preceded tone is tightly linked to an enhanced contractile capacity of ASM. We also have to acknowledge another important limitation of our study. We used isometric force as a surrogate for the contractile capacity of ASM ex vivo. Increased Rrs in response to MCh in vivo implies greater airway narrowing, which is more closely related to ASM shortening than isometric force. Force and shortening are indeed two different contractile properties that do not necessarily correlate (30). More studies are warranted to determine the effect of tone on the capacity of ASM to shorten.

Conclusions. Our findings stand along an emergent and very influential concept in the field of ASM contractility, which states that the force generated by ASM in response to a given contractile stimulus is not fixed (1, 7, 8, 42). By extension, the level of airway responsiveness obtained in vivo in response to a given dose of a contractile agonist can vary due to alterations in the contractile capacity of ASM. Our findings suggest that the prior state of ASM activation, which can be augmented by either a preceding dose of an inhaled spasmogen or the release of endogenous spasmogens (e.g., leukotrienes and histamine) in asthma can alter the level of airway responsiveness. Our finding carries two important messages for clinical and basic scientists who are conducting studies on airway responsiveness. The first message is one of awareness. We demonstrate

that preceding doses of a contractile agonist can affect the response to a subsequent dose by increasing the contractile capacity of ASM. Because the level of airway responsiveness is customarily tested by administering serial escalating doses of a contractile agonist, our findings raise the importance of standardizing the timing of dosage delivery during a specific bronchoprovocative challenge before comparing between groups or between two visits within the same individual. The second message is mechanistic. An elevated tone (confirmed by a greater than 10–12% reversibility of airway obstruction by a bronchodilator) and airway hyperresponsiveness (confirmed by an exaggerated response to delivered doses of a contractile agonist) are two pathognomonic features of asthma; so much that each of these features is henceforth used to diagnose asthma according to national and global guidelines (3, 16, 27, 33). However, whether a link of causality exists between them is still ill-defined. Our results suggest that an increased tone can augment airway responsiveness by increasing the contractile capacity of ASM. As such, an elevated tone may be one of the mechanisms by which in vivo defects found in asthma increase the contractile capacity of ASM and thereby increases airway responsiveness. Combined with others (1, 8), our findings represent new opportunities for tackling asthma because they pinpoint in vivo defects found in the lung of patients with asthma that are conducive to AHR by converting a normal ASM into a hypercontractile phenotype. Altogether, our study provides groundwork for further investigating 1) the intracellular pathways involved in the gain of force induced by tone, 2) the effect of other inflammatory spasmogens on the contractile capacity of ASM, and 3) the effect of tone on other contractile properties of ASM apart from isometric force, such as shortening.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.L.-G., D.G., M.-R.B., D.M., and Y.B. conception and design of research; A.L.-G. and D.G. performed experiments; A.L.-G. and Y.B. analyzed data; A.L.-G., M.-R.B., D.M., and Y.B. interpreted results of experiments; A.L.-G. and Y.B. prepared figures; A.L.-G. and Y.B. drafted manuscript; A.L.-G., D.G., M.-R.B., D.M., and Y.B. edited and revised manuscript; A.L.-G., D.G., M.-R.B., D.M., and Y.B. approved final version of manuscript.

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