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Interval between simulated deep inspirations on the dynamics of airway smooth muscle contraction in guinea pig bronchi

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ABSTRACT

A certain amount of time is required to achieve a maximal contraction from airway smooth muscle (ASM) and stretches of substantial magnitude, such as the ones imparted by deep inspirations (DIs), interfere with contraction. The duration of ASM contraction without interference may thus affect its shortening, its mechanical response to DIs and the overall toll it exerts on the respiratory system. In this study, the effect of changing the interval between DIs on the dynamics of ASM was examined *in vitro*. Isolated bronchi derived from guinea pigs were held isotonically and stimulated to both contract and relax, in a randomized order, in response to 10^{-5} M of methacholine and 10^{-6} M of isoproterenol, respectively. Interference to ASM was inflicted after 2, 5, 10 and 30 min in a randomized order, by imposing a stretch that simulated a DI. The shortening before the stretch, the stiffness before and during the stretch, the post-stretch elongation of ASM and the ensuing re-shortening were measured. These experiments were also performed in the presence of simulated tidal breathing achieved through force fluctuations. The results demonstrate that, with or without force fluctuations, increasing the interval between simulated DIs increased shortening and post-stretch elongation, but not stiffness and re-shortening. These time-dependent effects were not observed when ASM was held in the relaxed state. These findings may help understand to which extent ASM shortening and the regulatory effect of DI are affected by changing the interval between DIs. The potential consequences of these findings on airway narrowing are also discussed.

1. Introduction

The contraction of ASM is driven by several molecularprocesses. While some of these processes occur quickly upon contractile stimulation, such as the release of calcium from the sarcoplasmic reticulum, other processes progress over longer time-scales. A typical example is the rearrangement of the actin cytoskeleton, which is required for contraction and may take 5 to 15 min to fully develop (Mehta and Gunst, 1999; Tang, 2018). This indicates that a certain amount of time is required to achieve a maximal contraction.

The contraction of ASM is also influenced by many *in vivo* factors. Obvious examples include the molecules that are released endogenously and that actively relax ASM, such as adrenaline, nitric oxide and prostaglandin E_2. Mechanical factors, such as an imposed stretch, have also been widely studied and can seriously hamper ASM contraction (Fredberg et al., 1997; Gunst, 1983; Gunst et al., 1990). This is relevant to respiratory mechanics, as it implies that breathing maneuvers exert a bronchodilator effect by fluctuating the length of ASM (Fredberg et al., 1997).

The effect of stress and strain on the mechanics of ASM has become a topic of great interest over the last decades (Ansell et al., 2013, 2009; Fairbank et al., 2008; Fredberg et al., 1997; Gump et al., 2001; Gunst, 1983; Harvey et al., 2013; Ijpma et al., 2015; LaPrad et al., 2010, 2008; Lavoie et al., 2012; Noble et al., 2007; Oliver et al., 2007; Pascoe et al., 2013; Wang et al., 2000). Additional impetus to study this topic comes from the fact that the bronchoprotective and the bronchodilator effects of breathing maneuvers are impaired with aging (Scichilone et al., 2004a), as well as in patients suffering from asthma (Allen et al., 2008; Black et al., 2004; Burns et al., 1985; Fish et al., 1981; Jensen et al., 2001; Salome et al., 2003; Scichilone et al., 2001; Skloot et al., 1995; Slats et al., 2007), chronic obstructive pulmonary disease (Scichilone et al., 2008, 2004b; Slats et al., 2007) and obesity (Boulet et al., 2005; Holguin et al., 2010; Skloot et al., 2011). The search for the underlying mechanisms that govern the decline in contractility induced by stretching ASM has thus become a quest of great priority as it may unveil significant insights on the origin of respiratory symptoms in humans.

It is now clear that a certain threshold of strain, amounting to 0.75–3.3%, is required to perturb ASM contraction (Ansell et al., 2013; Harvey et al., 2013; Noble et al., 2007; Norris et al., 2015). Over this

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threshold, the bronchodilator effect of a stretch is proportional to its magnitude (Ansell et al., 2013; Harvey et al., 2013; Noble et al., 2007). Consequently, a stress of great magnitude, such as the one simulating a deep inspiration (DI), can generally strain the ASM sufficiently to trigger bronchodilation. The effect of fluctuating stress that simulated tidal breathing is more equivocal, as it has been shown to be sometime sufficient (Ansell et al., 2009; Fredberg et al., 1997; Gump et al., 2001; Harvey et al., 2013; LaPrad et al., 2008) and sometime insufficient (LaPrad et al., 2010; Lavoie et al., 2012; Noble et al., 2007) to attenuate ASM contractility.

Many factors also influence airway wall stiffness. For example, ASM tone (Lavoie et al., 2012; Noble et al., 2007; Pascoe et al., 2013), airway wall remodeling (Oliver et al., 2007) and hyperinflation (Cairncross et al., 2018; Hiorns et al., 2014) increase stiffness and may thereby limit the strain and the attendant bronchodilator effect of breathing maneuvers. Overall, the interplay between stress and strain on the mechanics of ASM with varying stiffness has been extensively studied (Ansell et al., 2013, 2009; Fairbank et al., 2008; Fredberg et al., 1997; Gump et al., 2001; Gunst, 1983; Harvey et al., 2013; Ijpma et al., 2015; LaPrad et al., 2010, 2008; Lavoie et al., 2012; Noble et al., 2007; Oliver et al., 2007; Pascoe et al., 2013; Wang et al., 2000). Contrastingly, the time over which the contraction is allowed to proceed without being interrupted by a mechanical interference has been largely overlooked (Mailhot-Larouche et al., 2017). Since time is required to achieve a maximal contraction, examining the effects of changing the interval between mechanical interferences on the shortening of ASM, as well as on its mechanical response to DIs, seems very important.

In the present study, isolated bronchi derived from guinea pigs were mounted in organ baths to assess the effect of changing the interval between stretches that simulated DIs on the dynamics of ASM. More specifically, the time elapsed since the previous stretch was changed during an ongoing contraction (or relaxation) to measure its impact on shortening and on the response to a subsequent stretch in terms of stiffness, post-stretch elongation of ASM and re-shortening. The experiments were conducted with or without constant force fluctuations to investigate the influence of simulated tidal breathing on the reported outcomes. The *in vivo* equivalent of this *in vitro* study would be to measure narrowing, the bronchodilator effect of DI and the re-narrowing after different durations of DI withholding during a persistent bronchoconstriction (or bronchodilation).

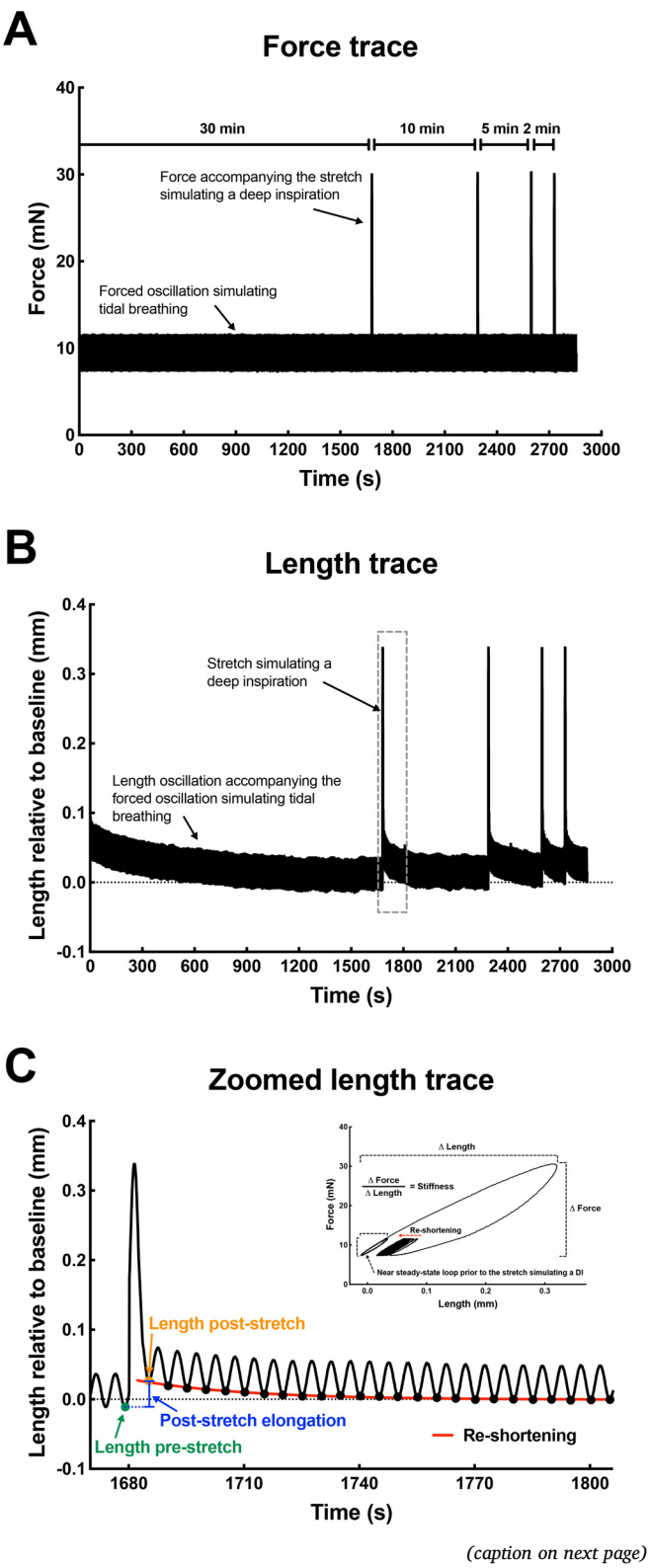
2. Methods

2.1. Animals

20-week-old female Hartley guinea pigs were used for these experiments. The protocol was approved by the Committee of Animal Care of *Université Laval* in accordance with the guidelines of the Canadian Council on Animal Care (Protocol 2015134-2).

2.2. Isolated bronchi

Guinea pigs were euthanized with pentobarbital sodium (110 mg/kg). The left main bronchus was excised and immersed in 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, 2.9 mM $CaCl_2$). Its length and diameter were measured. The movable side of two stainless steel triangles was then slipped into one end of the bronchial lumen and clipped back to its triangular shape on the other end in order to hold the bronchi in between two opposing triangles, each with one side passing through the lumen (Gazzola et al., 2016). The bronchus was held horizontally in a 40-ml organ bath between 2 platinum electrodes (2 mm wide × 50 mm long). The bath was filled with Krebs solution maintained at 37 °C by a jacketed bath containing circulating heated water. The upper triangle was connected by a surgical thread to a dual-mode lever arm system (model 300C; Aurora Scientific Inc., Aurora, Canada).



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The latter not only monitored force but also allowed excursions of both length and force to be applied. The lower triangle was connected to a stationary hook at the bottom of the bath. The resting tension was set by applying a distending force that simulated a transmural pressure at functional residual capacity (FRC) (*i.e.*, 5 cmH_2O) as previously described (Lee-Gosselin et al., 2013). Before starting the protocol described below, the bronchus was subjected to a period of equilibration of 60 min, during which time ASM was stimulated to contract every

Fig. 1. A representative force trace (**A**) and the corresponding length trace (**B**) of one bronchus in a constricted state. In each protocol, the bronchus was subjected to a series of four stretches of a magnitude simulating a deep inspiration. In this example, the time intervals between the stretches were set to 30, 10, 5 and 2 min, but the sequence of which was randomized. In this example, the bronchus was also subjected to force fluctuations to simulate tidal breathing. The force fluctuations (A) and the accompanying length fluctuations (B) cannot be seen at the time-scale shown and appear as thick lines. This protocol was repeated in four different conditions in a randomized order; with activated (methacholine at 10^{-5} M) and relaxed (isoproterenol at 10^{-6} M) ASM, both with and without force fluctuations in between stretches. A portion of the length trace in B (outlined by the dashed rectangle) is zoomed in **C**. The effect of changing the interval between stretches was assessed on: 1- the change in length pre-stretch; 2- the stiffness during the stretch; 3- the stiffness before the stretch; 4- the change in length post-stretch; 5- the post-stretch elongation; and 6- the re-shortening within the first 2 min after the stretch. The inset in C shows the length-force trace and how stiffness was calculated during the stretch and during the last simulated tidal breath before the stretch.

5 min for 20 s with an electrical field (60 Hz, 20 V, 2 ms) under isometric conditions.

2.3. Protocol

In each protocol, which lasted 47 min, the ASM within the bronchus was stretched on 4 occasions. The interval between stretches was set to 2, 5, 10 and 30 min in a random order (Fig. 1). The stretch simulated a DI from FRC to total lung capacity (TLC) in 2.5 s, as previously described (Mailhot-Larouche et al., 2017). It is important to understand that during the stretch, the system was controlling the length. This means that the length ASM attained during each stretch was identical regardless of ASM length before the stretch. Hence, the 'attained strain' during the stretch, and not the 'strain excursion', was controlled. Apart from during the stretch, the system was controlling the force. This means that ASM was held in isotonic conditions, which was set to a force equivalent to a transmural pressure of 5 cmH_2O. The ASM was thus allowed to freely adjust its length between the stretches. The protocol was performed with and without force fluctuations between stretches to simulate tidal breathing. More specifically, tidal breathing was simulated by oscillating the force sinusoidally at 0.2 Hz at an amplitude mimicking a transmural pressure from a trough at 5 cmH_2O to a peak at 10 cmH_2O.

The protocol, with and without force fluctuations, was also performed under two contractile states: 1- During contraction elicited by 10^{-5} M of methacholine; and 2- during relaxation elicited by 10^{-6} M of isoproterenol. Therefore, each bronchus was subjected to 4 randomized protocols: Contracted with methacholine or relaxed with isoproterenol with and without force fluctuations. The sequence of interventions that was used between protocols to reset history and to accustom ASM to its new contractile state, as well as the new conditions (with or without force fluctuations) under which it was operating, was described in detail previously (Mailhot-Larouche et al., 2017).

2.4. Data analyses

Data are presented as means ± standard errors (SE). For each bronchus, the position of the lever at the very beginning of the protocol in the presence of isoproterenol with no force fluctuations was set to zero. This represented the baseline. All the other positions adopted by the lever over the course of the experiment with the same bronchus were expressed in relation to this reference point. While negative values signify ASM shortening, positive values signify ASM lengthening. It is important to understand that the changes in length, reported in mm, truly refer to the changes in the position of the lever. It does not represent real changes in ASM length. The change in lever position is thus used as a surrogate for measuring the changes in ASM length. We believe it is a valid surrogate for ASM length as the direction toward which it changes is dictated by whether ASM is shortening or lengthening, and the magnitude by which it changes is related to real changes in ASM length.

The troughs during the force fluctuations, or the corresponding timepoints when there was no fluctuations, were used to quantify the changes in length between timepoints. This is to ensure that the comparisons between timepoints were all made at isoforce; herein to a force equivalent to a transmural pressure of 5 cmH_2O. Six variables were measured (Fig. 1): 1- The change in length immediately before the stretch; 2- the stiffness during the stretch, calculated as the change in force from trough to peak divided by the excursion of length over the same period; 3- the stiffness before the stretch, calculated as the change in force from trough to peak during the last simulated tidal breath prior to the stretch divided by the excursion of length over the same period (Obviously, these latter calculations could only be done when force fluctuations were applied); 4- the change in length immediately after the stretch; 5- the post-stretch elongation of ASM, measured as the difference in the changes in length immediately after and immediately before the stretch; and 6- the re-shortening during the first 2 min after the stretch. The effects of changing the interval between stretches and the contractile state (methacholine *versus* isoproterenol), as well as their interaction, on each of these variables were analyzed using repeated measures two-way ANOVAs. The same analyses were performed with and without force fluctuations. Statistical analyses were performed using Prism 7b (GraphPad Software, San Diego CA) and $p \leq 0.05$ was deemed significant.

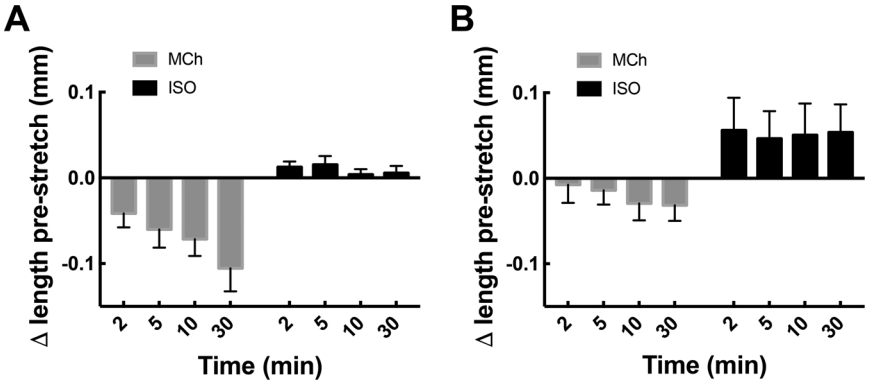

Fig. 2. The effect of changing the interval between stretches on ASM length immediately before the stretches. Guinea pig bronchi were appointed to two different contractile states in a randomized order. The contracted state (grey bars) was elicited by methacholine at 10^{-5} M and the relaxed state (black bars) was elicited by isoproterenol at 10^{-6} M. A series of stretches simulating deep inspirations was then imposed. The intercalated time between stretches was set to 2, 5, 10 and 30 min in a randomized order. The times depicted on the x-axis are the times elapsed since the previous stretch. Between stretches, the load opposing ASM was set to two different conditions in a randomized order. In one condition the load was fixed (**A**) and in the other condition the load was fluctuating to simulate tidal breathing (**B**). The length at which ASM settled at the very beginning of the protocol in the presence of isoproterenol with no force fluctuations was set to zero. The lengths reported on the graph are thus the changes relative to this baseline length. While negative values signify ASM shortening, positive values signify ASM lengthening. The effect of time was significant in both A ($p = 0.0009$) and B ($p = 0.05$). The interaction was significant in A ($p = 0.01$) but not in B ($p = 0.06$). $n = 7$.

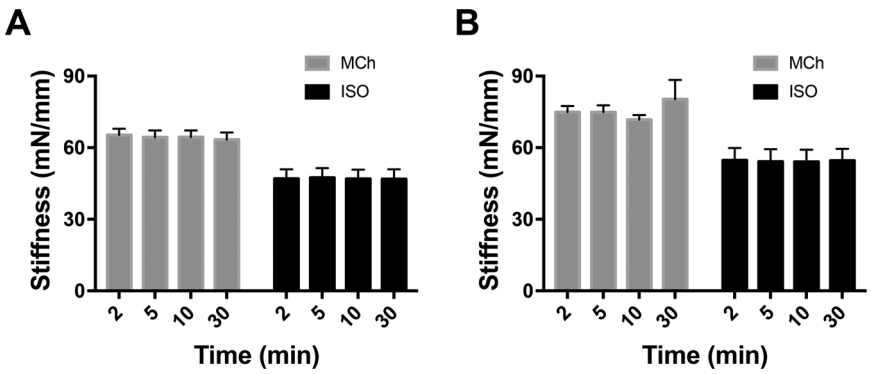

Fig. 3. The effect of changing the interval between stretches on airway wall stiffness during the stretches. The stiffness was measured as the excursion of force (mN) over the excursion of length (mm), as depicted in the inset of Fig. 1C. See the legend of Fig. 2 for a description of the graphs. n = 7.

3. Results

The effect of changing the interval between stretches on ASM length immediately before the stretches is depicted in Fig. 2. Increasing the interval between stretches increased ASM shortening. This was observed without (Fig. 2A; $p = 0.0009$) and with force fluctuations (Fig. 2B; $p = 0.05$). In conditions without force fluctuations, this time-dependent effect was also dependent on the contractile state of ASM, being observed in contracted state but not in relaxed state (the interaction being $p = 0.01$). Only a similar trend was observed in conditions with force fluctuations (interaction being $p = 0.06$ in Fig. 2B). By comparing Fig. 2A and Fig. 2B, within the same contractile state, one can also see the lengthening effect of force fluctuations ($p = 0.0001$).

The effect of changing the interval between stretches on airway wall stiffness is depicted in Fig. 3. Although the contractile state largely influenced stiffness both without (Fig. 3A; methacholine *vs* isoproterenol, $p = 0.004$) and with force fluctuations (Fig. 3B; methacholine *vs* isoproterenol, $p = 0.006$), the interval between stretches had no effect on stiffness. The force fluctuations also increased stiffness ($p < 0.0001$). This can be seen by comparing the same contractile state between Fig. 3A and Fig. 3B. The increased stiffness in conditions with force fluctuations may appear counterintuitive, but this is merely because ASM was at a longer length prior to the stretch (see Fig. 2A & B). The average length over which stiffness was measured was thus greater with force fluctuations and, consequently, the muscle appeared stiffer. This is because the increase in force caused by any given length excursion is greater when the ASM is operating further up in its force-length curve.

Stiffness was also measured during the last simulated tidal breath before the stretches (*i.e.*, at the near steady-state loop shown in the inset of Fig. 1C). The results are depicted in Fig. 4. As seen for the stiffness during the stretch, methacholine increased stiffness before the stretch ($p < 0.0001$). A clear trend towards an increase in stiffness by prolonging the intervals between stretches was also observed, but this effect was not significant ($p = 0.19$). There was also no interaction between the contractile state and the interval between stretches ($p = 0.29$).

The effect of changing the interval between stretches on ASM length immediately after the stretches is depicted in Fig. 5. The time-dependent increase in shortening observed before stretches in the contracted state (Fig. 2) was largely abolished after the stretches. However, it was still significant without force fluctuations (Fig. 5A; time, $p = 0.03$; interaction, $p = 0.03$), albeit not with force fluctuations (Fig. 5B; time, $p = 0.34$; interaction, $p = 0.22$). By comparing Fig. 5A and Fig. 5B, within the same contractile state, one can again see the lengthening effect of force fluctuations ($p = 0.003$).

The effect of changing the interval between stretches on the post-stretch elongation of ASM is depicted in Fig. 6. Increasing the interval between stretches increased ASM elongation induced by the stretches.

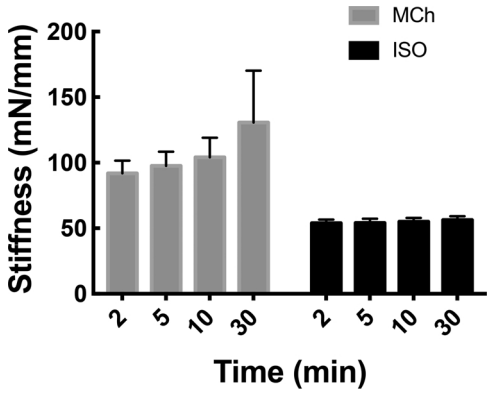

Fig. 4. The effect of changing the interval between stretches on airway wall stiffness before the stretches. The stiffness was measured as the excursion of force (mN) over the excursion of length (mm) during the last simulated tidal breath before the stretch (the near steady-state loop), as depicted in the inset of Fig. 1C. See the legend of Fig. 2B for a description of the graph. n = 7.

This was observed without (Fig. 6A; $p = 0.0001$) and with force fluctuations (Fig. 6B; $p < 0.0001$). This time-dependent effect was also dependent on the contractile state of ASM, being more pronounced in contracted state than in relaxed state (the interaction being $p = 0.02$ in Fig. 6A and $p = 0.005$ in Fig. 6B).

The effect of changing the interval between stretches on re-shortening after the stretches is depicted in Fig. 7. The re-shortening was calculated in percentage of the post-stretch elongation. Since the elongation was marginal in a relaxed state (Fig. 6A & B), re-shortening was only calculated in the contracted state. Increasing the interval between stretches markedly decreased re-shortening. However, this is only because the elongation of ASM induced by stretches increased with longer intervals (Fig. 6A & B). When expressed in absolute values, re-shortening was not affected by changing the interval between stretches, both without (inset of Fig. 6A) and with (inset of Fig. 6B) force fluctuations. The force fluctuations had no significant effect on re-shortening. This can be seen by comparing Fig. 7A and Fig. 7B.

4. Discussion

This study demonstrates that increasing the interval between simulated DIs greatly enhanced ASM shortening. This study also confirms that a stretch of a magnitude simulating a DI reset ASM to an elongated length, which may in part explain the bronchodilator effect of a DI (Fredberg et al., 1997). This study also shows that force fluctuations simulating tidal breathing lengthened ASM. Finally, this study demonstrates that the extra shortening achieved during longer intervals between simulated DIs was to a great extent reversed by the simulated DI, at least in our setting where the attained strain during the simulated

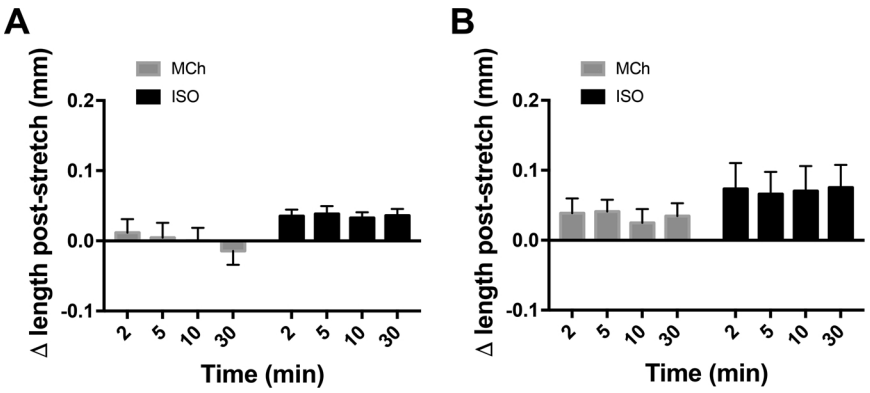

Fig. 5. The effect of changing the interval between stretches on the change in ASM length immediately after the stretches. The effect of time was significant in A ($p = 0.02$) but not in B ($p = 0.34$). The interaction was significant in A ($p \leq 0.03$) but not in B ($p = 0.22$). See the legend of Fig. 2 for a description of the graphs. n = 7.

DI was controlled. As a result, the post-stretch elongation of ASM (*i.e.*, the bronchodilator effect of DI) was progressively increasing as the interval between the simulated DIs was increasing. Together, these findings have important implications in respiratory mechanics. More specifically, our *in vitro* results would predict that increasing the interval between DIs does not affect airway wall stiffness but alarmingly increases airway narrowing. Fortunately, tidal breathing seems to exert a protective effect. It also seems that as long as the airway wall reaches a given maximal strain during the DI, the bronchodilator effect of DI would increase with the extent of narrowing. It should thereby reset respiratory function almost to the same superior level irrespective of the time spent without taking a DI.

The majority of *in vitro* studies investigating the contraction of ASM are conducted under isometric conditions. This is chiefly because it is easier. It is then assumed that force should translate *in vivo* to ASM shortening and thus airway narrowing and respiratory symptoms. Although this line of reasoning is logical, it is obviously not a substitute to real experimental data. Our study was conducted in a setting that allows ASM to freely shorten when its force exceeds the load against which it is acting (or to freely lengthen when the load is exceeding its force). We also performed the experiments without or with force fluctuations to account for the dynamic environment within which ASM normally resides and operates.

The main goal of the present study was to assess the effect of changing the interval between simulated DIs on ASM shortening and on the response to a simulated DI. In order to control for the attained strain during the simulated DI, we decided to control the length, even though the force was controlled for the rest of the experiments. In our first attempt to study the effect of changing the interval between simulated DIs on ASM dynamics, we conducted our experiments in conditions where the oscillations of lengths were controlled during both the simulated tidal breathing and the DIs (Mailhot-Larouche et al., 2017). The results obtained with these different methodologies are described in the following paragraphs.

The progressive shortening that we observed in the present study with incremental intervals between simulated DIs would have not been predicted based on our previous study. Indeed, when the system was controlling the length for the entire experiments, the force generated by ASM was not affected by changing the interval between simulated DIs (Mailhot-Larouche et al., 2017). It is possible that the length oscillations that was used to simulate tidal breathing in this previous study, *versus* the conditions without and with force fluctuations used in the present study, might have prevented the gain in contraction overtime. Alternatively, the assumption that force and shortening always change in conjunction may not be correct, as they conspicuously respond differently to the same intervention (*i.e.*, changing the interval between stimulated DIs) in our two studies.

The lack of change in stiffness during the stretch was consistently observed in both studies. This suggests that the ease by which the contracted ASM is strained in response to a simulated DI is not affected by changing the interval between simulated DIs. It also suggests that stiffness was not affected by the extent of shortening, as the latter was progressively increasing with incremental intervals between simulated DIs in the present study. It is worth mentioning that the excursion of strain inflicted by the stretch in the present study was augmenting with incremental intervals. This is because shortening was increasing during longer intervals and that the attained strain, and not the strain excursion, was controlled. Therefore, the lack of change in stiffness observed in the present study indicates that the excursion of force measured during the stretch was increasing with the same proportion as the excursion of strain.

The post-stretch elongation of ASM, which is the *in vitro* equivalent of the bronchodilator effect of a DI, was augmenting with incremental intervals (Fig. 6). Contrastingly, our previous study predicted that changing the interval between simulated DIs did not affect the bronchodilator effect of a simulated DI (Mailhot-Larouche et al., 2017). We believe that this discrepancy resulted from the variable strain excursions inflicted by the stretch in the present study. As

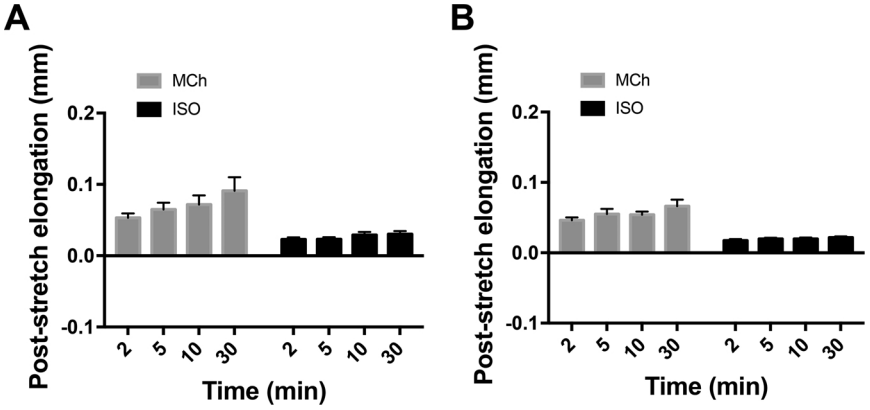

Fig. 6. The effect of changing the interval between stretches on the post-stretch elongation of ASM. Fig. 1C illustrates where this elongation was calculated and what it represents. The effect of time was significant in both A ($p = 0.0001$) and B ($p < 0.0001$). The interaction was significant in both A ($p = 0.01$) and B ($p = 0.005$). See the legend of Fig. 2 for a description of the graphs. n = 7.

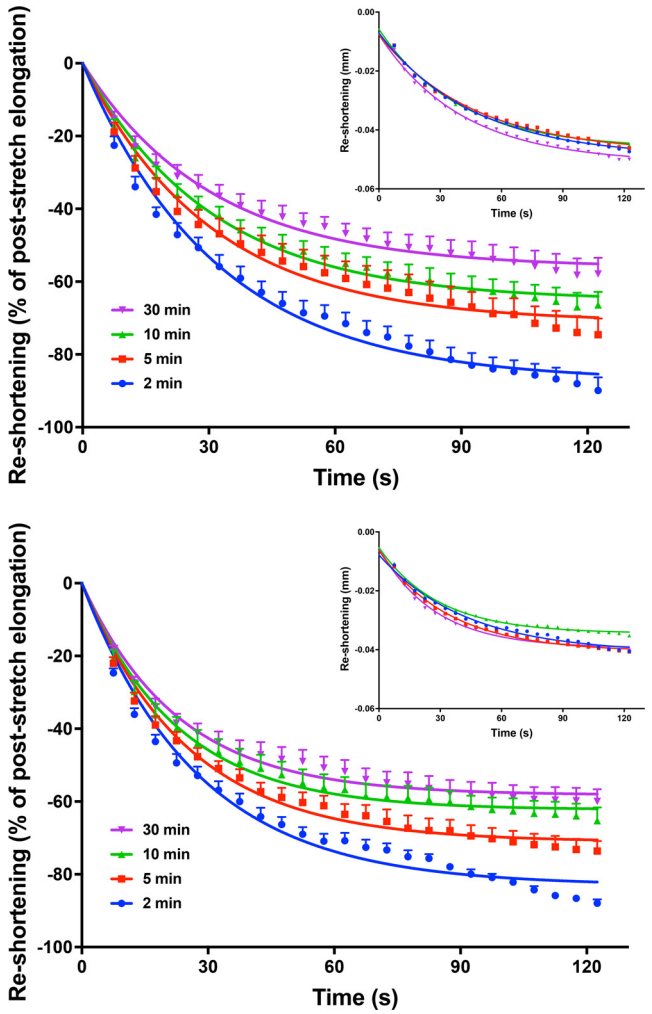

Fig. 7. The effect of changing the interval between stretches on ASM re-shortening. These bronchi were contracted with methacholine at 10^{-5} M. **A** and **B** were performed without and with force fluctuations, respectively. The blue, red, green and violet curves are for elapsed times since the previous stretch of 2, 5, 10 and 30 min, respectively. The re-shortening within the first 2 min after the stretch is expressed in percentage of the post-stretch elongation (Fig. 6). n = 7.

aforementioned, the increased shortening observed with longer intervals implies that greater strain excursions were inflicted by the stretch. It is very likely that these greater strain excursions were accountable for the greater post-stretch elongation of ASM. This interpretation is uncertain and discussed further below. Interestingly, the length at which ASM settled after the stretch was always the same, at least during force fluctuations, irrespective of the duration of the interval between simulated DIs and the consequent shortening achieved. Hence, the greater post-stretch elongation of ASM observed at longer intervals between simulated DIs occurred due to a smaller length before the stretch, and not because ASM settled at a longer length after the stretch. This result also seems to suggest that the attained strain during the stretch dictated the length at which ASM settled after the stretch. More studies will be required to ascertain this last statement.

The re-shortening post-stretch, which is the *in vitro* equivalent of the re-narrowing post-DI, was not affected by changing the interval between simulated DIs. This was somewhat unexpected. Based on our previous study, it was predicted that the re-narrowing post-DI slows down as the interval between DIs increases (Mailhot-Larouche et al., 2017). This apparent discrepancy is discussed below. One nuance is important to point out though. Since the rate of re-shortening was unaffected by changing the interval between simulated DIs and that the post-stretch elongation of ASM was increasing with time, the re-shortening to pre-stretch length was quicker after shorter intervals between simulated DIs. This would be consistent with our previous conclusion (Mailhot-Larouche et al., 2017).

A huge advantage of working *in vitro* is that any confounder, or combination of confounders, can be controlled. Many outcomes can also be measured all at once. However, interpreting the results of sequential outcomes, especially when they are interdependent, is sometime difficult. Herein, we opted for controlling the force during the simulated tidal breathing and for controlling the length during the intermittent stretches that simulated DIs. Interpreting the effect of our intervention (*i.e.*, changing the interval between simulated DIs) on the degree of shortening is pretty straightforward. This merely represents measuring the effect of an intervention on the first outcome. However, measuring the effect of our intervention on post-stretch ASM elongation is not as simple. In our setting, the increased shortening observed with longer intervals also increased the excursion of strain during the stretch. A greater post-stretch elongation of ASM may thus have arisen due to our intervention, to a greater strain excursion or to the combined effect of both.

The same applies for the subsequent outcomes, namely the re-shortening, which may have been affected by our intervention but also by the magnitude of strain excursion and the extent of post-stretch elongation. Thus, standing alone, our study provides little opportunities to interpret the effect of changing the interval between simulated DIs on re-shortening. Be that as it may, a tentative explanation can be provided for the apparent discrepancy between our two studies concerning the effect of changing the interval between simulated DIs on the predicted rate of re-narrowing. Ansell and coworkers have demonstrated that increasing the dilator effect of DI increases the rate of re-narrowing (Ansell et al., 2013). So while longer intervals were expected to decrease the rate of re-narrowing based on the results of our first study (Mailhot-Larouche et al., 2017), the increased post-stretch elongation observed after longer intervals between simulated DI in the present study was expected, based on Ansell's study (Ansell et al., 2013), to increase the rate of re-shortening. It thus seems that our intervention and the result of a preceding outcome (*i.e.*, the bronchodilator effect of DI) acted in opposite direction on the subsequent outcome (*i.e.*, the rate of re-narrowing) and may ultimately cancel each other out. Together, this also points to the importance of using different methodologies to decipher the respective and collective contributions of both the intervention and the first outcomes on the subsequent outcomes.

Although not the primary goal of the present study, our results suggested that breathing at tidal volume exerts a bronchodilator effect. Indeed, the length of ASM was clearly longer with force fluctuations than without fluctuations irrespective of the interval between simulated DIs and the contractile state. This was somewhat unexpected, as the bronchodilator effect of tidal breathing is controversial (Ansell et al., 2009; Fredberg et al., 1997; Gump et al., 2001; Harvey et al., 2013; LaPrad et al., 2010, 2008; Lavoie et al., 2012; Noble et al., 2007). Harvey and coworkers have demonstrated that the bronchodilator effectiveness of tidal fluctuations is in great part dictated by the effective compliance of the airways (Harvey et al., 2013). More specifically, they showed that airways subjected to fluctuating transmural pressure from 1 cmH_2O dilate more than the same airways subjected to the same transmural fluctuations but from 5 cmH_2O. They suggested that a smaller starting pressure allows the airways to operate on a more compliant part of the pressure-radius curve and thereby dilate more because the strain inflicted by the fluctuating pressure is then greater (Harvey et al., 2013). In the present study, the force acting against ASM was the same without force fluctuations than at the trough during force fluctuations (equivalent of 5 cmH_2O). A change in effective compliance is thus unlikely to account for the effect we observed. Other factors may have contributed. Interestingly, Wang and coworkers have demonstrated that apart from the amplitude of stain, the time over which the fluctuations are applied is a strong predictor of the bronchodilator

effect of simulated tidal breathing (Wang et al., 2000). Since the periods over which force fluctuations were imposed in our experiments were long (10 to 57 min), it may explain why we observed such a marked effect of simulated tidal breathing on ASM length.

In conclusions, increasing the interval between simulated DIs significantly increased ASM shortening. This phenomenon occurred under both a fixed and a fluctuating isotonic load, although ASM always settled at longer lengths in the presence of force fluctuations. Interestingly, when the attained strain is controlled during the simulated DI, the extra shortening observed with incremental intervals was for the most part reversed by the simulated DI. Inasmuch as the results of *in vitro* experiments are translatable to *in vivo* observations, our study pointed to an important factor that may substantially impact the response to inhaled methacholine. In fact, our findings predict that longer is the interval of contraction without interference, more likely it is to adversely affect the respiratory system. This is relevant to the seminal work of Malmberg and coworkers, showing that reducing the interval between DIs decreased airway responsiveness *in vivo* in humans (Malmberg et al., 1993). Conveniently, based on our findings, the extra shortening caused by increasing the interval between simulated DIs seems to be largely counteracted by the subsequent DI, at least when a given maximal airway wall strain is attained. These latter findings are relevant to King and coworkers' study (King et al., 1999), showing that the increased narrowing caused by longer intervals before the first DI post-methacholine challenge is to a great extent reversed by subsequent DIs (King et al., 1999). Overall, this *in vitro* study dissected out the impact of changing the intervals between stretches on ASM shortening, as well as on the regulatory effect of stretch on shortening. These responses may help to understand the role played by ASM in determining the degree of airway responsiveness and the respiratory relief afforded by a DI. Owing to the important contribution of airway hyperresponsiveness and the impaired bronchodilator effect of DI in the manifestation of respiratory symptoms, this study may also shed light on the origin of respiratory disorders.

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