Pharmacological bronchodilation is partially mediated by reduced airway wall stiffness

T. K. Ansell[1], P. B. Noble[1, 2], H. W. Mitchell[1], and P. K. McFawn[1]

Author Contributions

T. K. Ansell performed the organ bath experiments, morphometry and prepared the manuscript. P. B. Noble, H. W. Mitchell and P. K. McFawn provided intellectual input into study design, data interpretation and contributed to manuscript preparation. All animal handling was performed by T. K. Ansell and P. B. Noble.

[1]School of Anatomy, Physiology and Human Biology, University of Western Australia, Crawley, WA, Australia. [2]Centre for Neonatal Research and Education, School of Paediatrics and Child Health, University of Western Australia, Crawley, WA, Australia.

Correspondence should be addressed to:

Thomas K. Ansell

School of Anatomy, Physiology and Human Biology

M311

University of Western Australia

35 Stirling Hwy

Crawley, WA, 6009

Australia

Phone: 618 6488 3310

Fax: 618 6488 1025

E-mail: Thomas.Ansell@uwa.edu.au.au

Running Head: Oscillation is synergistic with bronchodilators

Summary

Background and purpose: In asthmatic patients, airflow limitation is at least partially reversed by administration of pharmacological bronchodilators, typically β_2-agonists. In addition to receptor-mediated bronchodilation, the dynamic mechanical environment of the lung itself can reverse bronchoconstriction. We have now explored the possibility that bronchodilators exert a synergistic effect with oscillatory loads by virtue of reducing airway wall stiffness and therefore, enhancing the bronchodilatory response to breathing manoeuvres.

Experimental approach: Whole porcine bronchial segments *in vitro*, were contracted to carbachol and relaxed to the non-specific β-agonist, isoprenaline, under static conditions or during simulated breathing manoeuvres.

Key results: The bronchodilatory response to isoprenaline was greater during breathing manoeuvres, compared with the response under static conditions. Since the bronchodilatory response to breathing manoeuvres is dependent on airway smooth muscle (ASM) strain and therefore, airway wall stiffness, our findings are likely explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain, producing greater bronchodilation.

Conclusions and implications: A contribution of reduced airway stiffness and increased ASM strain to the bronchodilator action of isoprenaline is shown, suggesting that oscillatory loads act synergistically with pharmacologically mediated bronchodilation. The implications for the

treatment of asthma are that reducing airway wall stiffness represents a potential target for novel pharmacological agents.

Keywords: airway smooth muscle, asthma, bronchoconstriction, deep inspiration, isoprenaline and strain

Abbreviations: ACh, Acetylcholine; AHR, Airway hyper-responsiveness; $A_{i,}$ Area enclosed by the internal area; A_{mo}, Area enclosed by the outer ASM perimeter; ASM, Airway smooth muscle; cAMP, Cyclic 3', 5' adenosine monophosphate; CCh, Carbachol; DI, Deep inspiration; DRC, Dose-response curve; LABA, Long-acting β_2-agonist; MRLC, Myosin regulatory light chain; NO, Nitric oxide; PD_2, -logEC_{50}; P_{mo}, Outer ASM perimeter; P_{tm}, Transmural pressure; WA_i, Inner wall area

Introduction

Bronchoconstriction is initiated by agonist interaction with G-protein coupled receptors (Barnes, 1989), which through an intracellular cascade, phosphorylates myosin regulatory light chain (MRLC) and facilitates actin and myosin binding and airway smooth muscle (ASM) contraction. Excessive bronchoconstriction (i.e. airway hyper-responsiveness, AHR) is a major contributor to airflow limitation (Lambert *et al.*, 1982), and a primary characteristic of asthma. In asthmatic patients, airflow limitation is at least partially reversed by administration of pharmacological bronchodilators, typically β_2-agonists. Agonist binding of β_2-receptors on ASM activates adenylyl cyclase, which produces an increase in cyclic 3', 5' adenosine monophosphate (cAMP) and de-phosphorylation of MRLC (Barnes, 1995). In isolated ASM strips (Gump *et al.*, 2001) and whole bronchial segments (Ansell *et al.*, 2009a) *in vitro*, β_2-agonists relax ASM in a dose-dependent manner and *in vivo*, increasing dose of β_2-agonists produces greater improvement in FEV_1 (Barnes *et al.*, 1983).

In addition to receptor-mediated bronchodilation, the dynamic mechanical environment of the lung itself can reverse bronchoconstriction. In normal healthy individuals, deep inspirations (DI) produce a transient bronchodilation (Duggan *et al.*, 1990; Hida *et al.*, 1984; Nadel *et al.*, 1961; Salerno *et al.*, 2005). The underlying mechanism by which DI produces bronchodilation is not completely understood but likely involves strain-induced (i.e. length-change) reversal of ASM force due to perturbed cross-bridge binding (Fredberg *et al.*, 1999; Fredberg *et al.*, 1997) and/or de-polymerisation of the contractile filaments (Gunst *et al.*, 1995). As the magnitude of strain applied to the ASM is increased with deeper depth of inspiration, there is increasing

bronchodilation (Ansell *et al.*, 2013; Lavoie *et al.*, 2012; Salerno *et al.*, 2005). Lesser inspirations, such as normal tidal breathing, may also produce some bronchodilation, but which are strongly dependent on the stiffness of the airway wall, as a stiffer airway wall will reduce ASM strain and therefore bronchodilation (Harvey *et al.*, 2013; LaPrad *et al.*, 2008).

Airway wall stiffness is clearly dependent on the structural composition of the airway (Noble *et al.*, 2002). However, it is also clear that a major contributor to stiffness is the degree of tension present in the ASM (Kelly *et al.*, 2012; Vincent *et al.*, 1970). Muscle contraction markedly increases stiffness (Hubmayr *et al.*, 1996; Noble *et al.*, 2007), whilst ASM relaxants markedly reduce airway stiffness (Ansell *et al.*, 2009a; Hubmayr *et al.*, 1996). Given the importance of airway wall stiffness to the bronchodilator efficacy of breathing manoeuvres discussed above, it has been mooted that some proportion of the bronchodilation produced by ASM relaxants, including β-agonists, is attributable to their effect on airway stiffness (Ansell *et al.*, 2009a). That is, pharmacological bronchodilators should be expected to enhance the effectiveness of breathing manoeuvres at producing bronchodilation.

The effects of pharmacological bronchodilators and dynamic ASM strain have been compared previously in whole bronchial segments subjected to fixed-volume oscillation (Ansell *et al.*, 2009a) and in isolated ASM strips using fixed-length oscillation (Gump *et al.*, 2001). We showed that bronchodilation produced by the combined effect of pharmacological bronchodilators (including isoprenaline) and ASM strain (i.e. volume oscillation) was not greater than bronchodilation to either alone, suggesting that the pharmacological and physiological

mechanisms producing bronchodilation were not synergistic but separate (Ansell *et al.*, 2009a). Similar conclusions were reached in the earlier study on isolated ASM strips subject to length oscillation (Gump *et al.*, 2001). However, because the ASM strain was held fixed in our former study and in the study by Gump and colleagues, any effect of isoprenaline-induced airway softening on ASM strain and bronchodilation could not be identified.

We have now built on previous work in our laboratory (Ansell *et al.*, 2009a) to determine if there is indeed an enhancement of oscillation induced bronchodilation by β-agonists due to changes in airway wall stiffness. Porcine whole bronchial segments *in vitro* were contracted to carbachol (CCh) and relaxed to the non-specific β-agonist, isoprenaline, under static conditions or during simulated breathing manoeuvres. An important adaptation of previous approaches (Ansell *et al.*, 2009a) was to simulate breathing manoeuvres by oscillating airway wall transmural pressure (P_{tm}), as occurs in lungs *in vivo* under physiological conditions. Under these conditions the magnitude of ASM strain produced by simulated breathing manoeuvres will be dependent on airway wall stiffness, allowing β-agonists to modify oscillatory induced bronchodilation through its effects on airway wall stiffness. Present results show the previously postulated synergism between isoprenaline and ASM strain regimens, which was not detected by earlier fixed-ASM strain protocols. Our findings support an important role of pharmacological bronchodilators in mediating the bronchodilatory response to breathing manoeuvres by reducing airway wall stiffness.

Methods

Animal handling

All animal experiments conformed to institutional ethics and animal care unit regulations (Animal Ethics Committee, University of Western Australia, Crawley, WA, Australia). Male White Landrace pigs, ~35 kg, were initially sedated with tiletamine-zolazepam (4.4 mg/kg^{-1} *I.M.*) and xylazine (2.2 mg/kg^{-1} *I.M.*) and then exsanguinated under sodium pentobarbitone anaesthesia (30 mg/kg^{-1} *I.V.*). The lungs were removed and transported on ice to the laboratory.

Airway segment preparation

Bronchial segments were dissected from the main stem bronchus of the left or right lower lobe. All side branches were ligated with surgical silk and a ~28 mm long airway segment was cannulated at both ends, as previous described (Ansell *et al.*, 2013; Ansell *et al.*, 2009a; Ansell *et al.*, 2009b). Briefly, the mode generation was 17 at the distal and 11 at the proximal end (where trachea = 0), with an internal diameter of ~2 mm at the distal and ~3 mm at the proximal end. Following cannulation, the airway was mounted horizontally in an organ bath containing gassed (95 % O_2 and 5 % CO_2) Krebs solution (121 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5 mM sodium morpholinopropane sulfonic acid, 11.5 mM glucose and 2.5 mM $CaCl_2$; pH 7.3) at 37 °C. The length of the segment was stretched to 105 % of its length in the fully deflated lung, shown previously to approximate the length at functional residual capacity (Noble *et al.*, 2005).

The proximal end of the airway lumen was connected to a reservoir filled with Krebs solution, the height of which set the initial P_{tm} (5 cmH_2O) and which was used to flush the lumen with

Krebs solution between experiments. The distal end of the airway was connected to a liquid filled syringe pump. The syringe pump was capable of simulating breathing manoeuvres in 1-of-2 ways: fixed-P_{tm} oscillations or fixed-volume oscillations (see below). All protocols were performed in a closed system, created by closure of a tap between the airway and the Krebs solution reservoir. The system was leak free with negligible compliance (0.0113 μL/cmH_2O with a ~7.0 mL system volume).

Simulation of breathing manoeuvres

A custom-built servo-controlled syringe pump and pressure transducer were used to measure airway narrowing and to apply fixed-P_{tm} oscillations (i.e. breathing manoeuvres), as previously described (Ansell *et al.*, 2013; Noble *et al.*, 2013; Noble *et al.*, 2011). Briefly, airways were connected to a 1 mL glass syringe driven by a feedback-controlled servomotor and motor controller and P_{tm} was measured *via* a calibrated pressure transducer with feedback to the servomotor. Changes in airway luminal volume (i.e. airway narrowing and fixed-P_{tm} oscillations) were measured *via* a calibrated displacement transducer that measured the rotation of the syringe motor. Using this approach, P_{tm} was set to the desired level (i.e. static or oscillatory, see *Experimental protocols*) and ASM activation resulted in a decrease in lumen volume (i.e. airway narrowing, Figure 1A). Measurement of ASM force and fixed-volume oscillations were applied using the same syringe pump oscillator described above but using the displacement transducer and not pressure transducer as the feedback control to the servomotor. Using this approach, lumen volume does not decrease in response to ASM activation but instead results in an increase in P_{tm} (i.e. active pressure) that represents ASM force production (Figure 1B). For comparisons with protocols that used fixed-P_{tm} oscillations, the volume of oscillation

(i.e. breathing manoeuvres) was that which produced the same trough to peak change in P_{tm} in the contracted state (i.e. at the peak of contraction following the administration of the contractile agonist) and was fixed thereafter.

Experimental protocols

After dissection and mounting, airways were initially equilibrated to organ bath conditions for ~60 min under a static P_{tm} of 5 cmH_2O, approximating the mechanical environment present at functional residual capacity, *in vivo*. Viability of the tissue was confirmed through stimulation with acetylcholine (ACh, 10^{-4} M) added to the organ bath. Airways were subsequently contracted to a single dose of CCh (10^{-6} M) under both static (5 cmH_2O P_{tm}) and oscillatory conditions in a randomised order. For the fixed-P_{tm} approach, the oscillatory protocol comprised continuous large breathing manoeuvres (Δ15 cmH_2O at 0.25Hz). For the fixed-volume approach, the volume changes used were adjusted for each airway so that breathing manoeuvres were Δ15 cmH_2O in the contracted state. The initial volume change needed to produce a Δ15 cmH_2O after contraction was approximated from previously published experiments (Noble *et al.*, 2007). After the contraction plateaued, the oscillation volume was adjusted (if needed) to give a Δ15 cmH_2O pressure swing. Tissues were oscillated for 6 min before contraction to CCh. Once contraction to CCh had plateaued, full dose-response curves (DRC) were constructed to the non-specific β-agonist, isoprenaline (10^{-7} to 3x 10^{-5} M). Experiments conducted using the fixed-P_{tm} or fixed-volume approaches were performed in separate groups of airways.

Morphometry

Morphometric analyses were carried out to estimate the magnitude of ASM strain produced by breathing manoeuvres, as previously described (Ansell *et al.*, 2013). Briefly, following experimentation, airways were removed from the organ bath and fixed in 4 % formaldehyde solution under atmospheric pressure (i.e. 0 cmH_2O P_{tm}). Distal and proximal regions of the airway segment were processed into paraffin blocks. Transverse airway sections were cut at a thickness of 5 μm and stained with haematoxylin and eosin. Inner wall area (WA_i) was calculated from the area enclosed by the outer ASM perimeter (A_{mo}) minus the area enclosed by the internal area (A_i) (Bai *et al.*, 1994) using ImageJ (version 1.45j, National Institutes of Health, MD, U.S.A.). Measurements at distal and proximal locations were averaged and corrected for horizontal stretch (105 % of its length in the fully deflated lung), which reduces the cross sectional area of the wall, assuming tissue volume is constant. The calculated inner wall area was also corrected for tissue shrinkage that occurs during histological processing (Ansell *et al.*, 2013; Noble *et al.*, 2013).

Analysis and statistics

Lumen volume (i.e. prior to the administration of CCh) was measured by the volume that could be withdrawn until closure in the relaxed airway at 5 cmH_2O P_{tm} (Gunst *et al.*, 1988). Airway narrowing to CCh (for the fixed-P_{tm} approach) was expressed as % lumen volume (where 100 % airway narrowing indicates airway closure). As described above, morphometry allowed the outer ASM perimeter (P_{mo}) to be calculated using the following equation:

$$P_{mo} = \sqrt{4 \times \pi \times \left(WA_i + \frac{\text{Lumen Volume}}{\text{Airway Length}}\right)}$$

Where, lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement and airway length is the length of the airway segment mounted in the organ bath. The equation assumes WA_i is constant at all P_{tm}, that P_{mo} is circular and that the lumen is cylindrical. Active pressure to CCh (for the fixed-volume approach) was expressed as ΔP_{tm}. Comparisons between static and oscillatory conditions were made at troughs of the oscillation cycle (volume or pressure, depending on the approach used). The response to isoprenaline was also expressed as % of the response to CCh (i.e. % contracted). Dose-response curves expressed as % contracted had variable slope sigmoidal curves fitted to individual airways. Sensitivity ($PD_2 = -\log_{10}(EC_{50})$) to isoprenaline were calculated for individual airways under static and oscillatory conditions. During fixed-P_{tm} oscillations, ASM strain was calculated as $\Delta P_{mo}/P_{mot}$, where ΔP_{mo} is the tough to peak change in P_{mo} during the breathing manoeuvre and P_{mot} is the trough P_{mo} immediately prior to the breathing manoeuvres.

Specific compliance of the airway wall was calculated from the Δvolume in relation to the ΔP_{tm} during the inflationary limb of the tidal oscillation cycle using the equation:

$$\text{Specific Compliance} = \frac{\Delta\text{Volume}}{\Delta P_{tm} \times \text{Lumen Volume}}$$

Where Δvolume and ΔP_{tm} are the trough to peak changes in volume and pressure during the breathing manoeuvre and lumen volume is volume of the lumen at the trough of the pressure cycle at the time of measurement.

Differences between groups were analysed using 2-way repeat measures ANOVA and Newman-Keuls *post hoc* test with dose of isoprenaline and the condition (i.e. either static or oscillatory) as the repeat measures variables, unless otherwise stated below. The response to CCh under static and oscillatory conditions and the sensitivity to isoprenaline under static and oscillatory conditions was analysed using paired t-tests. Data analysis and statistical tests were performed using Statistica (version 8.0; StatSoft, Tulsa, OK, U.S.A.) and GraphPad Prism (version 5.0d; GraphPad Software, La Jolla, CA, U.S.A.) Data are presented as means ± SEM, where n = number of animals.

Results

Under static conditions, CCh (10^{-6} M) produced 59.2 ± 4.9 % narrowing (Figure 2A) and 46.2 ± 2.5 cmH_2O active pressure (Figure 2B). Isoprenaline (10^{-7} to 3x 10^{-5} M) reversed airway narrowing and active pressure in a dose-dependant manner. At the maximum dose of isoprenaline, airway narrowing fell to 39.1 ± 3.4 % narrowing and active pressure fell strongly to 2.6 ± 1.8 cmH_2O under static conditions. Interestingly, when expressed as % of the response to CCh, the maximum reversal of active pressure with isoprenaline was greater than the maximum reversal airway narrowing ($p<0.001$).

Airways also stiffened strongly in response to CCh ($p<0.001$). Specific compliance of the airway wall fell from 0.0126 ± 0.0013 cmH_2O^{-1} in the relaxed state, to 0.0037 ± 0.0003 cmH_2O^{-1} following CCh for the fixed-P_{tm} approach. Similarly, for the fixed-volume approach, specific compliance to fell from 0.0099 ± 0.0011 cmH_2O^{-1} in the relaxed state, to 0.0014 ± 0.0002 cmH_2O^{-1} following CCh. Isoprenaline reduced airway stiffness in a dose-dependent manner for both the fixed-P_{tm} and fixed-volume approaches (Figure 3). Specific compliance increased to 0.0111 ± 0.0011 cmH_2O^{-1} and 0.0065 ± 0.0007 for the fixed-P_{tm} and fixed-volume approaches, respectively.

The magnitudes of oscillations (i.e. ΔP_{tm} or Δvolume) used were chosen so that contraction prior to the administration of isoprenaline was not substantially attenuated. Airway narrowing and active pressure fell to 47.7 ± 5.3 % (Figure 4A) and 39.4 ± 3.7 cmH_2O (Figure 4B) (compared to 59% and 46 cmH_2O, see above) during fixed-P_{tm} and fixed-volume oscillations, respectively.

There was no difference in sensitivity (PD_2) to isoprenaline under static, compared with oscillatory, conditions (Table 1).

By comparing the bronchodilatory response to isoprenaline under static and oscillatory conditions, we sought to determine whether β_2-agonists exerted a secondary bronchodilator effect by virtue of reducing airway wall stiffness and therefore, enhancing the bronchodilatory response to breathing manoeuvres. Our results demonstrate that the bronchodilatory response to isoprenaline was greater during fixed-P_{tm} oscillations, compared with the response under static conditions (Figure 5A). At maximal dose of isoprenaline, airway narrowing fell to 10.4 ± 2.6 % (i.e. a ~82 % reversal from contracted) during breathing manoeuvres but to only 39.1 ± 3.4 % (i.e. a ~35 % reversal from contracted) under static conditions. The greater bronchodilatory response to isoprenaline during fixed-P_{tm} oscillations is likely explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain (Figure 5B), producing greater bronchodilation. Airway smooth muscle strain produced by fixed-P_{tm} oscillations increased from 0.03 ±.0.003 (i.e. a 3 % increase in ASM perimeter) to 0.08 ± 0.007 (i.e. a 8 % increase in ASM perimeter) to isoprenaline in a dose-dependent manner.

In contrast to the experiments where P_{tm} oscillations were held fixed, under conditions where fixed-volume oscillations were applied, ASM strain was constant for each airway at 0.01 ± 0.002 (i.e. a 1 % increase in ASM perimeter) and therefore, independent of changes in airway wall compliance produced by isoprenaline. Consequently, there was no difference in the response to isoprenaline under static, compared with oscillatory, conditions (Figure 6). At maximal dose of

isoprenaline, active pressure fell to 2.6 cmH_2O (i.e. a ~97 % reversal from contracted) during breathing manoeuvres and 2.3 cmH_2O (i.e. a ~98 % reversal from contracted) under static conditions.

Discussion and conclusions

The present study determined whether pharmacological bronchodilators produce part of their physiological action through reduction of airway stiffness that enhances the relaxation produced by oscillatory loads. We show that the bronchodilatory response to isoprenaline was greater during simulated breathing manoeuvres, compared with the response under static conditions. We propose that the greater bronchodilatory response to isoprenaline during breathing manoeuvres is likely explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain, producing greater bronchodilation.

In the present study, we used our established intact bronchial segment model. Our laboratory has previously modelled tidal breathing and DI manoeuvres in both animal (Ansell *et al.*, 2013; Noble *et al.*, 2007; West *et al.*, 2012) and human (Noble *et al.*, 2011) bronchial segments, including those from subjects with reported asthma (Noble *et al.*, 2013). The aforementioned studies simulate breathing manoeuvres by varying airway P_{tm}. In the present study, the applied fixed-P_{tm} oscillations modelled breathing manoeuvres (Δ15 cmH_2O), larger than normal tidal breathing but less than a DI (Δ25 cmH_2O). We assume that during bronchoconstriction, *in vivo*, P_{tm} would increase above that occurring with normal tidal breathing in order to overcome the greater resistance of the respiratory system and to maintain minute ventilation. We induced ~59 % airway narrowing, which we calculate, assuming laminar flow, to be produce a substantial five to six-fold increase in airway resistance.

In order to establish a hypothesised synergy of isoprenaline and oscillation, we compared the bronchodilatory response to isoprenaline under static conditions and during breathing manoeuvres simulated by oscillating P_{tm}. Isoprenaline produced greater bronchodilation (i.e. reversal of airway narrowing) during fixed-P_{tm} oscillations, with increasing separation from the static control with increasing dose of isoprenaline, suggesting a synergistic relationship. Since the bronchodilatory response to breathing manoeuvres is dependent on ASM strain (Ansell *et al.*, 2013; Noble *et al.*, 2007) and therefore, airway wall stiffness, our findings are likely explained by the effect of isoprenaline on reducing airway wall stiffness, which increased ASM strain. As discussed below, synergism was only revealed when oscillations of fixed-P_{tm} were used, whereas fixed-volume oscillations did not alter the response to isoprenaline.

Studies from our laboratory (Ansell *et al.*, 2009a) and others (Gump *et al.*, 2001) have previously examined the combined effect of pharmacological bronchodilators when the strain applied to the ASM (length-change in isolated ASM strips or lumen volume in airway segments) is held fixed. The principal conclusions drawn from these studies was that ASM length/volume oscillation and pharmacological bronchodilators act *via* separate pathways. That is, while both oscillation and pharmacological bronchodilators produced ASM relaxation, one did not affect the other. However, in our prior study (Ansell *et al.*, 2009a) we proposed one caveat: that pharmacological bronchodilators through their actions on reducing airway stiffness could theoretically maximise the strain-induced relaxation of ASM force, which we have now demonstrated by administering isoprenaline under fixed-P_{tm} conditions. To further examine the mechanism underlying the greater bronchodilatory response to isoprenaline during simulated breathing manoeuvres, we again compared the relaxant response to isoprenaline during fixed-volume oscillations. Under

conditions where fixed-volume oscillations were applied, and ASM strain was constant and therefore independent of changes in airway wall compliance produced by isoprenaline, there was no synergism between oscillatory and pharmacological pathways. This finding supports our proposal that the greater bronchodilatory response to isoprenaline during fixed-P_{tm} oscillations (i.e. synergism) was mediated by reduced airway wall stiffness.

The increased drug efficacy during P_{tm} oscillation is unlikely to be unique to isoprenaline, nor β_2-agonists in general, rather, any pharmacological bronchodilator that reduces airway wall stiffness is predicted to undergo a similar synergy. We have previously shown in whole bronchial segments (Ansell *et al.*, 2009a) *in vitro*, that nitric oxide (NO) also reverses ASM stiffness, and ASM cell stiffness is reduced in culture by numerous bronchodilator agonists such as isoproterenol, prostaglandin E_2 and forskolin amongst others (Hubmayr *et al.*, 1996). The synergy between pharmacological bronchodilation and breathing stresses is considered 'mechanical' through its dependence on ASM stiffness.

With the exception of the specific β_2-agonist, salbutamol, in 1962 (Cullum *et al.*, 1969) and the long-acting β_2-agonist (LABA), salmeterol, in 1988 (Ullman *et al.*, 1988), pharmacological bronchodilators have remained largely unchanged for 60 years. Not all patients with asthma respond well to current bronchodilator therapy (Wenzel, 2006). Our finding that, at maximal dose, about half of the pharmacological bronchodilator effect is mediated by reduced airway wall stiffness has clinical implications for the treatment of asthma. Reducing airway wall stiffness represents a potential target for novel pharmacological agents (Bossé *et al.*, 2010; Raqeeb *et al.*,

2012; Seow, 2012). Drug design models that consider the agonist's effects on the reversal of both ASM force and stiffness should lead to more effective pharmacological intervention.

Airway wall compliance and ASM strain were somewhat greater during fixed-P_{tm}, compared with fixed-volume, oscillations. There are several possible explanations. Since the airway narrows in the fixed-P_{tm} approach, the airway wall may operate at a more compliant region of the pressure-volume curve. In the pig, the airway wall is most compliant below 5 cmH_2O P_{tm} (at least in the relaxed state), before stiffening again at –5 cmH_2O (Noble *et al.*, 2002). In comparison, the airway does not narrow in the fixed-volume approach and therefore, may operate at a comparatively stiffer region of the pressure volume curve. This explanation is not entirely sufficient, as there was a tendency for lower specific compliance during fixed-volume oscillations in the relaxed state. An alternative explanation is that the initial higher pressure swings in the fixed-P_{tm} approach facilitated greater wall compliance. The volume oscillations were chosen such that pressure swings following the administration of CCh matched the Δ15 cmH_2O in the fixed-P_{tm} protocol, which meant that in the relaxed state pressure swings accompanying fixed-volume oscillations were considerably less (<4 cmH_2O). The reduction in airway wall stiffness produced by isoprenaline also differed between the fixed-P_{tm} and fixed-volume approaches, where the increase in compliance was greater during fixed-P_{tm} oscillations, which may be explained by further 'softening' of the airway wall due to greater ASM strain.

The amplitudes of fixed-P_{tm} and fixed-volume oscillations in the present study were chosen such that bronchoconstriction was not substantially attenuated by the breathing manoeuvres alone.

Fixed-P_{tm} oscillations only modestly attenuated airway narrowing (~81 % of the response under static conditions), and there was a non-significant tendency towards reduced active pressure during volume oscillation (~85 % of the response under static conditions). During oscillations, the compliance of the airway wall determined the magnitude of ASM strain. Prior to the administration of isoprenaline, ASM strain during fixed-volume oscillations was ~1 %, compared with ~3 % during fixed-P_{tm} oscillation, due to the difference in compliance between the fixed-P_{tm} and fixed-volume approaches. Somewhat serendipitously, prior studies including those from our own laboratory suggest that strain between 1 - 3% are necessary to affect the contractile apparatus (Ansell *et al.*, 2013; Fredberg *et al.*, 1997; Harvey *et al.*, 2013; Noble *et al.*, 2007). Therefore, we are confident that the amplitudes of fixed-P_{tm} and fixed-volume oscillations in the present study were sufficient to examine the bronchodilatory response to isoprenaline and ASM strain.

Several other interesting and potentially important aspects of this study require discussion. During fixed-volume oscillations, active pressure was completely reversed by high doses of isoprenaline, however, airway wall stiffness had not returned to levels present in the relaxed state. Several studies have shown that, in response to contractile agonists, ASM stiffens prior to generating active force (Ansell *et al.*, 2013; Pascoe *et al.*, 2012). Indeed, in whole bronchial segments, the sensitivity to acetylcholine is greater with respect to airway wall stiffening than active force or airway narrowing (Ansell *et al.*, 2013). The use of a sub-maximal dose of CCh in the present study likely produced a proportionally greater increase in airway wall stiffness than ASM contraction (i.e. airway narrowing or active pressure). The observation that isoprenaline was not able to completely reverse airway wall stiffening, compared with active force, may be

due to the fact that there was more airway wall stiffening to reverse (note that the highest isoprenaline dose was chosen to produce maximum reversal of active force and airway narrowing, rather than airway wall stiffness). A similar disconnect between stiffness and airway narrowing during fixed-P_{tm} oscillations was not observed, which may also be explained by further 'softening' of the airway wall due to greater ASM strain. Nonetheless, a scenario in which airway wall stiffening occurs prior to narrowing means that, during exacerbation of asthma, the bronchodilatory response to breathing manoeuvres becomes less effective early in the process.

Finally, our results showed that under static conditions, reversal of active pressure by isoprenaline was greater than the corresponding reversal of airway narrowing. As there was no difference in the dose of CCh or isoprenaline between protocols, we assume that cell signalling was comparable. However, ASM mechanics will be different at the level of the contractile apparatus, since ASM shortens in the fixed-P_{tm} approach whilst in the fixed-volume approach the muscle contracts isometrically (i.e. no shortening). Airway smooth muscle is responsive to length-change, a phenomenon termed 'length adaptation' (Bossé *et al.*, 2008; Seow, 2005) and it has been suggested that prolonged ASM shortening facilitates greater contraction (McParland *et al.*, 2005). In the present study, in the experiments where we measured airway narrowing, length adaptation may have occurred, producing a reduced bronchodilatory response to isoprenaline. Whilst the present study cannot provide any further explanation as to why the bronchodilatory response to isoprenaline may be less effective in the experiments where we measured airway narrowing, the implication of these findings are that drug design models which measure ASM force, rather than airway narrowing may overestimate the effectiveness of pharmacological

bronchodilators. A disconnect between ASM force and airway narrowing may also, at least in part, explain the discrepancies between experiments in isolated ASM strips and *in vivo* responses to breathing manoeuvres (Lutchen, 2013).

In conclusion, the present study found that, at maximal dose at least half of the bronchodilator effect of β_2-agonists is mediated by reduced airway wall stiffness. To our knowledge, this is the first time that a secondary effect of a pharmacological bronchodilator has been experimentally shown and which is likely to be of clinical significance. The implications for the treatment of asthma are that reducing airway wall stiffness represents a potential second target for novel pharmacological agents.

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Figure Legend

Table 1. PD_2 to isoprenaline (10^{-7} to 3x 10^{-5} M) under static and oscillatory conditions.

	Static	**Oscillatory**
Fixed-P_{tm} Approach	**Airway Narrowing**	**Airway Narrowing**
	5.60 ± 0.11	6.00 ± 0.07
Fixed-Volume Approach	**Active Pressure**	**Active Pressure**
	6.26 ± 0.14	7.78 ± 1.53

There was no difference in the sensitivity to isoprenaline for airway narrowing or active pressure under static, compared with oscillatory conditions. n = 6. Mean ± SEM.

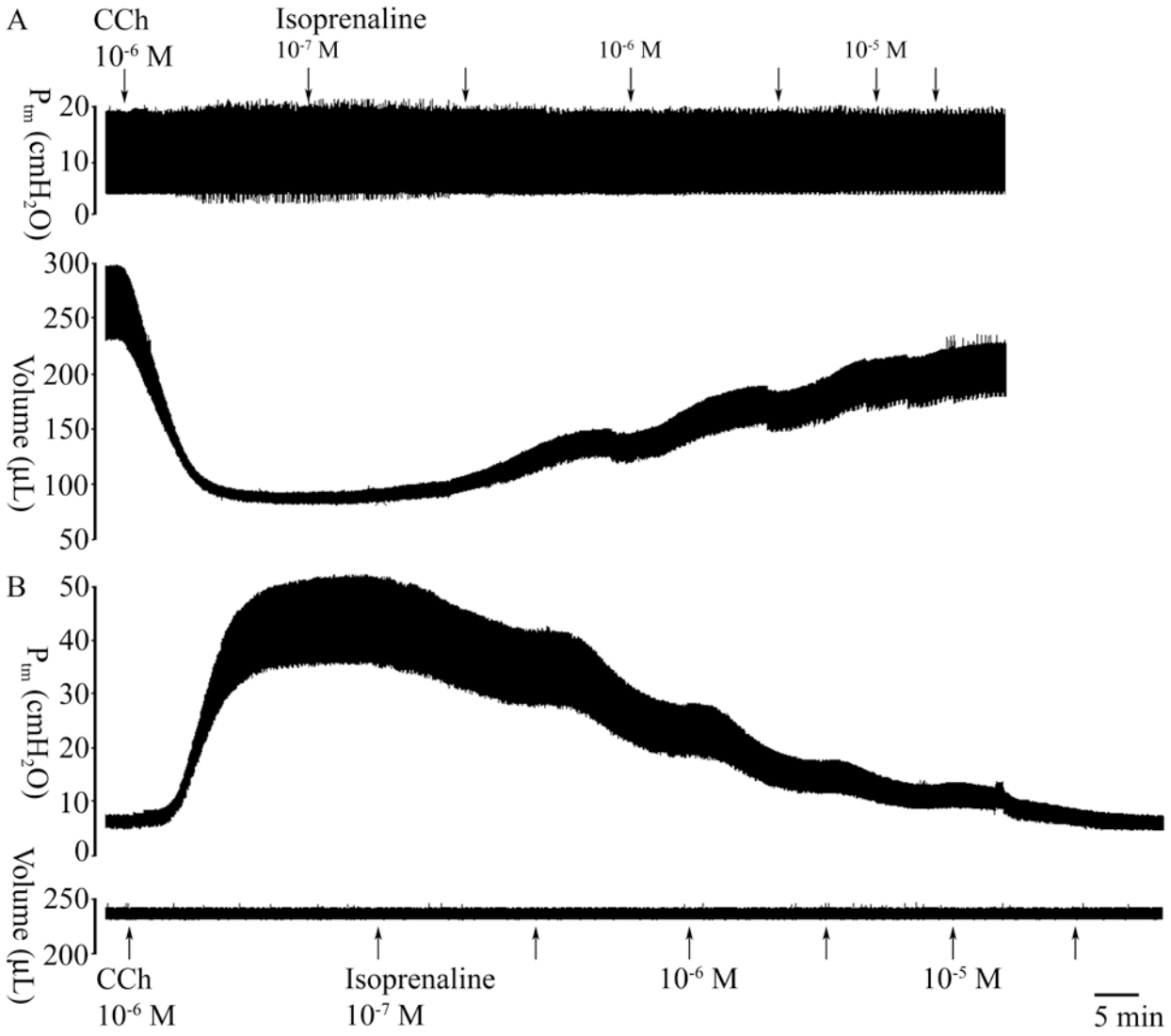
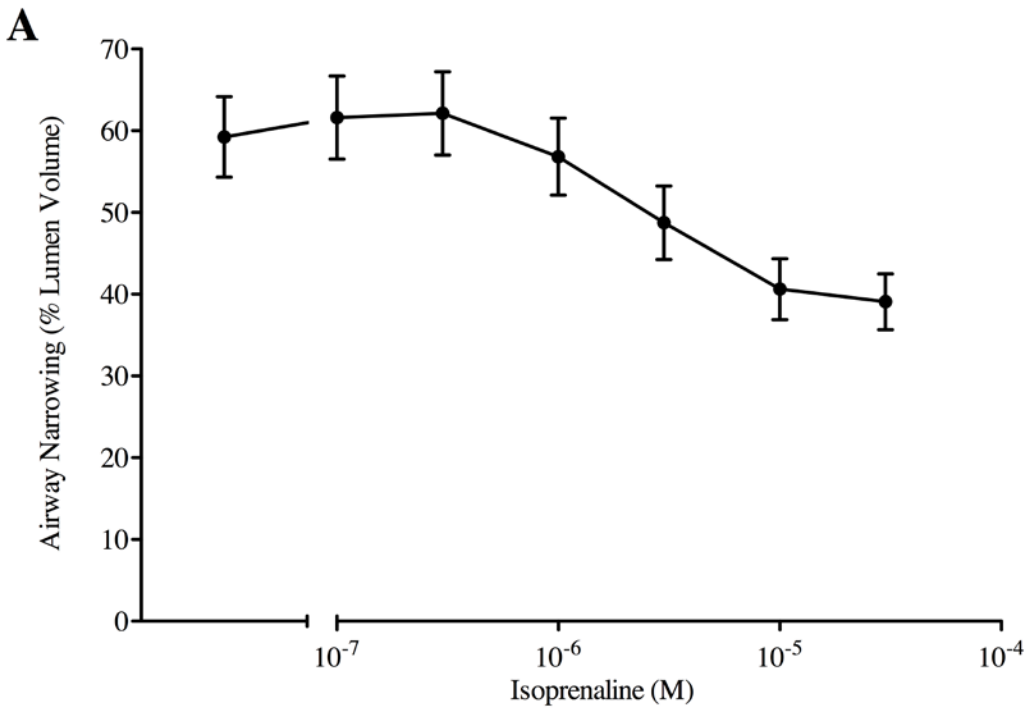


Figure 1. An example trace of a cumulative dose-response curve (DRC) to isoprenaline (10^{-7} to 3x 10^{-5} M, *arrows*, text labels shown only for whole log doses) using fixed-transmural pressure (P_{tm}, A) and fixed-volume (B) oscillations in airways contracted to carbachol (CCh, 10^{-6} M). At the time scale shown, individual oscillations are not visible but appear as a thick line, the thickness of which indicates the magnitude of the P_{tm} and volume oscillations. In response to isoprenaline, lumen volume increased during fixed-P_{tm} oscillations and P_{tm} decreased during fixed-volume oscillations, in a dose-dependant manner. Dose-response curves were performed under static conditions (trace not shown) and during continuous large breathing manoeuvres.


A
Airway Narrowing (% Lumen Volume)
70
60
50
40
30
20
10
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Isoprenaline (M)

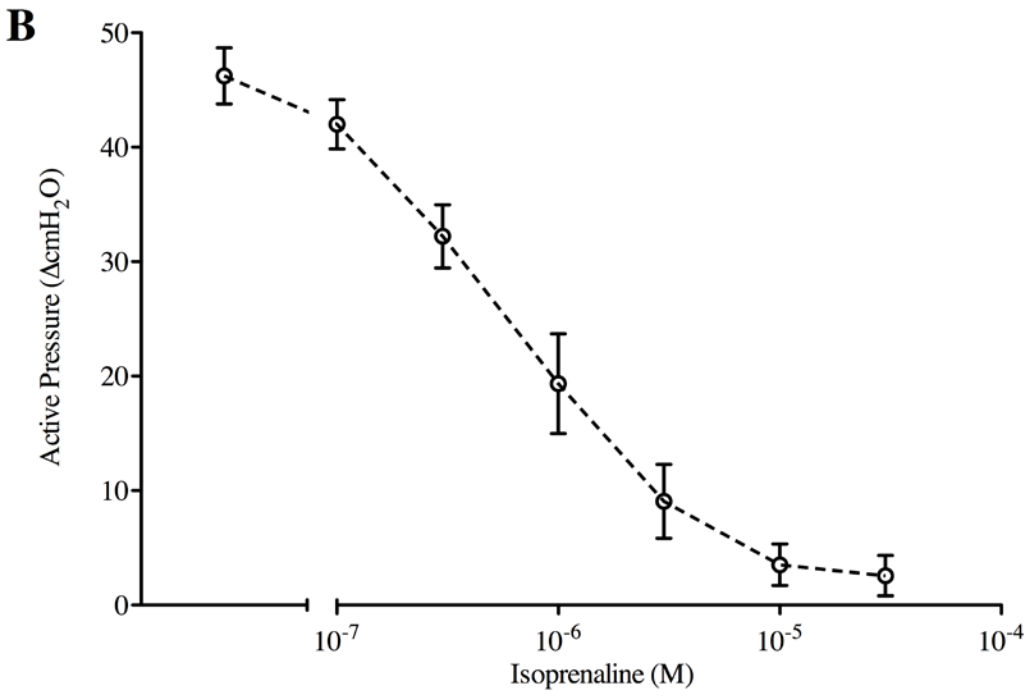

B
Active Pressure (ΔcmH_2O)
50
40
30
20
10
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Isoprenaline (M)

Figure 2. Cumulative DRC to isoprenaline (10^{-7} to 3x 10^{-5} M) under static conditions for the fixed-P_{tm} (% Lumen Volume, A) and fixed-volume (ΔcmH_2O, B) approaches in airways contracted to CCh (10^{-6} M, left of the axis break). Isoprenaline reversed airway narrowing ($p<0.001$) and active pressure ($p<0.001$) in a dose-dependant manner. n = 6. Mean ± SEM.

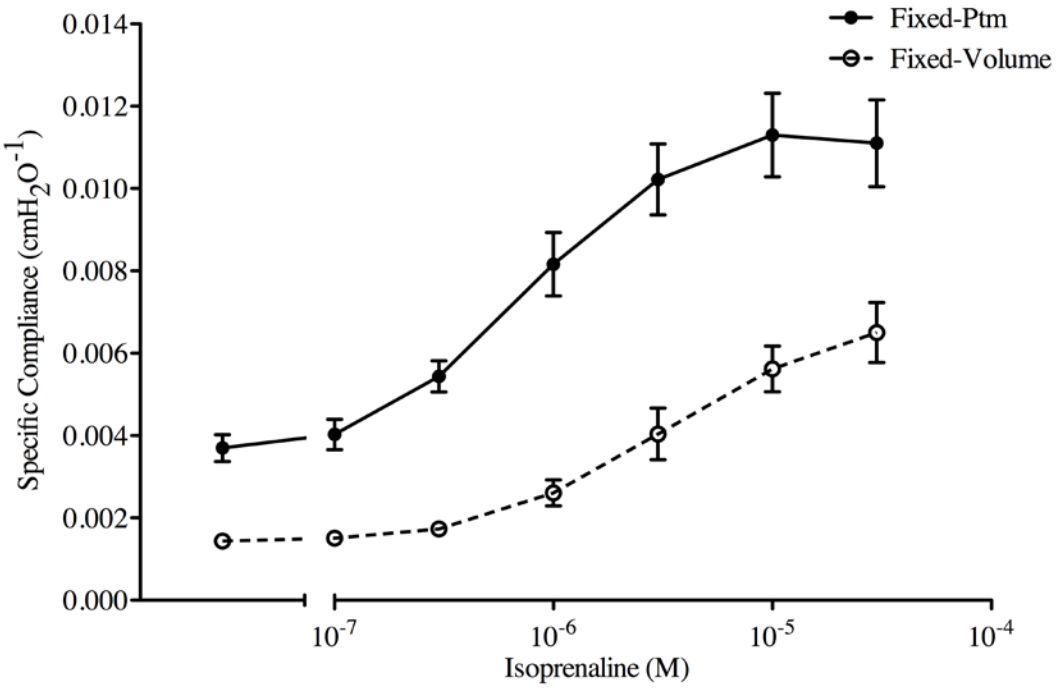


Figure 3. Specific compliance of the airway wall (cmH_2O^{-1}) in response to isoprenaline (10^{-7} to 3x 10^{-5} M) in airways contracted to CCh (10^{-6} M, left of the axis break). Isoprenaline reduced airway wall stiffness in a dose-dependant manner for both the fixed-P_{tm} ($p<0.001$) and fixed-volume ($p<0.001$) approaches. Airways were stiffer for the fixed-volume, compared with the fixed-P_{tm}, approach ($p<0.001$). n = 6. Mean ± SEM.

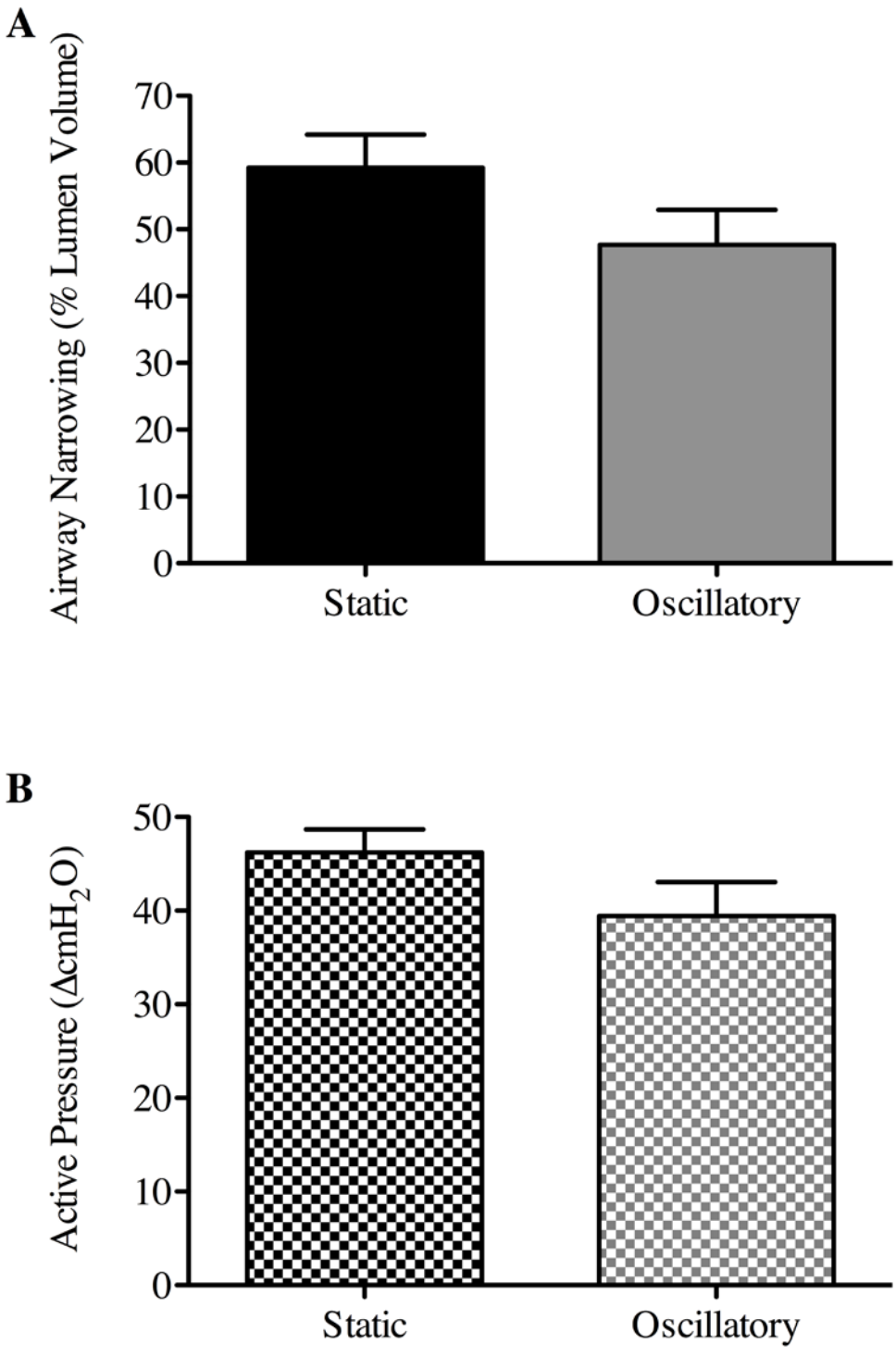
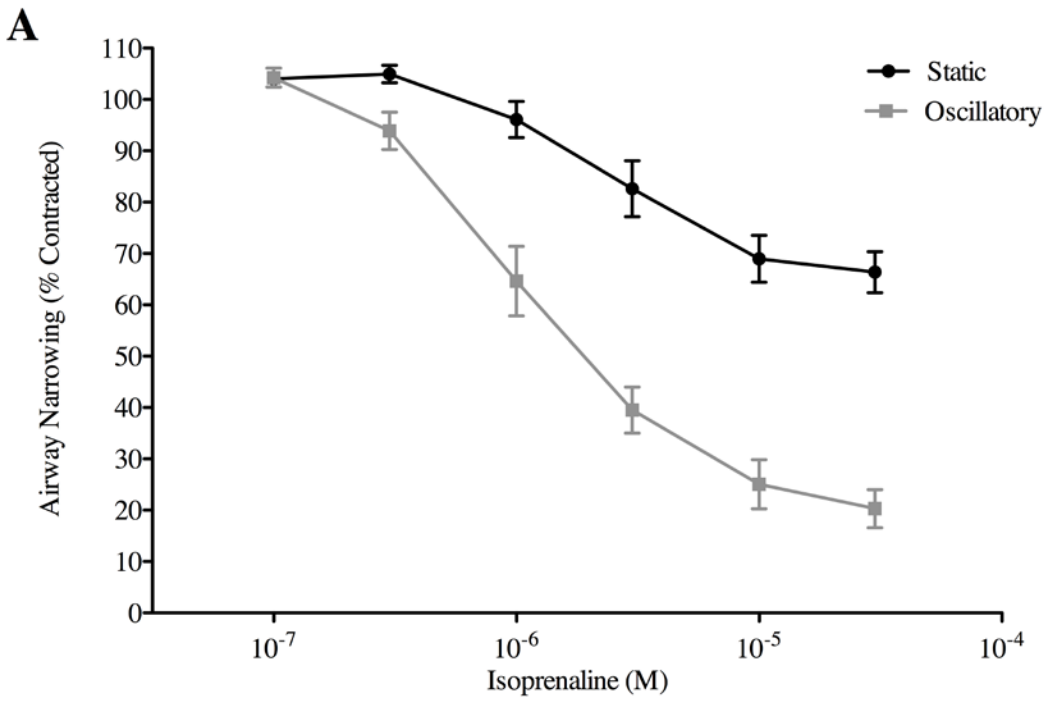


Figure 4. Airway narrowing (% Lumen Volume, A) and active pressure (ΔcmH_2O, B) to CCh (10^{-6} M) under static (black) and oscillatory (grey) conditions. Fixed-P_{tm} oscillations attenuated

airway narrowing ($p<0.05$). There was also a tendency towards a reduction in active pressure with fixed-volume oscillation, however, this did not reach statistical significance ($p=0.06$). $n = 6$. Mean ± SEM.


A
Static
Oscillatory
Airway Narrowing (% Contracted)
110
100
90
80
70
60
50
40
30
20
10
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Isoprenaline (M)

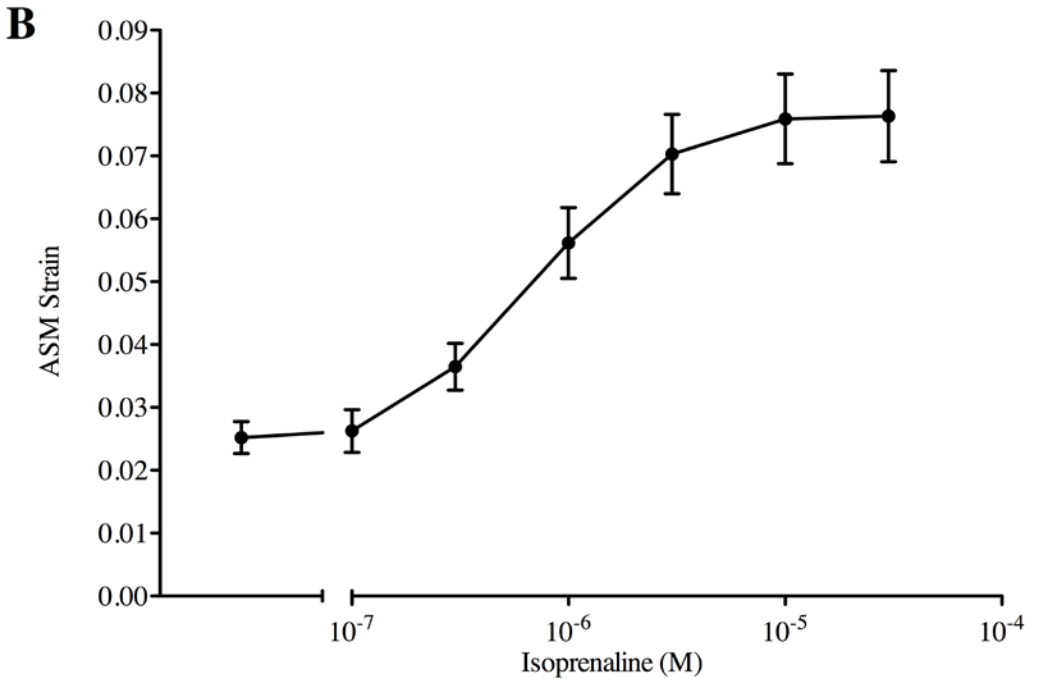

B
ASM Strain
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Isoprenaline (M)

Figure 5. Cumulative DRC to isoprenaline (10^{-7} to 3x 10^{-5} M) under static conditions and using fixed-P_{tm} oscillations (% Contracted, A) and airway smooth muscle (ASM) strain (B) produced by fixed-P_{tm} oscillations in airways contracted to CCh (10^{-6} M, left of the axis break in B). Isoprenaline enhanced the response to fixed-P_{tm} oscillations ($p<0.001$). Airway smooth muscle strain produced by fixed-P_{tm} oscillations increased in a dose-dependant manner ($p<0.001$). Dose-response curves under static conditions in A are the same data as in Figure 2A but expressed as a % of contraction. n = 6. Mean ± SEM.

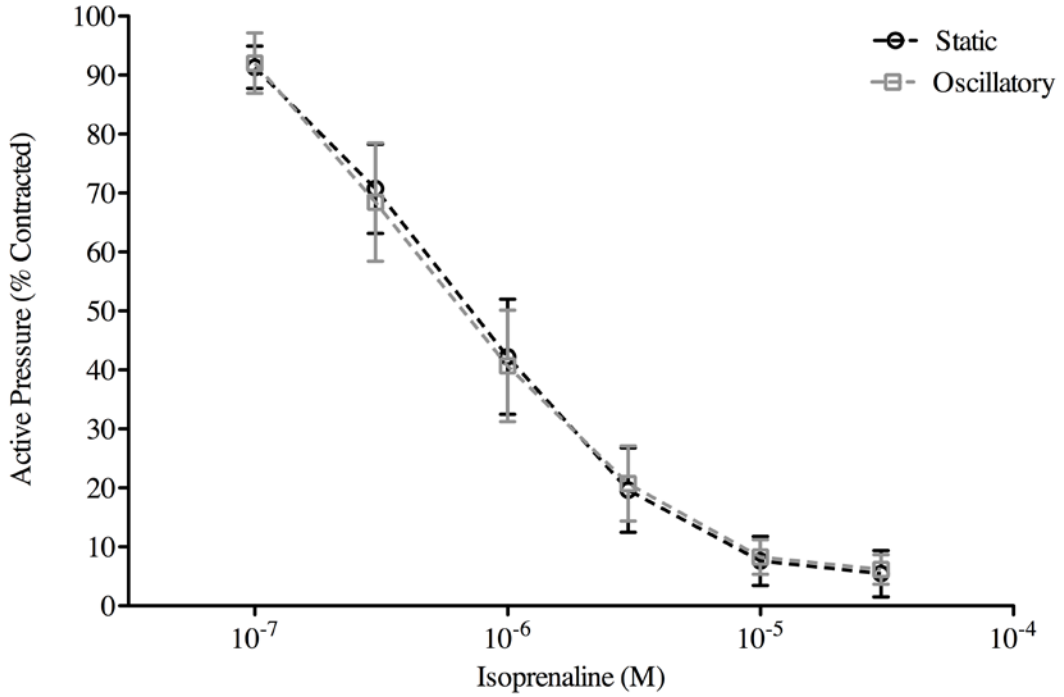


Figure 6. Cumulative DRC to isoprenaline (10^{-7} to 3x 10^{-5} M) under static conditions and using fixed-volume oscillations (% Contracted) in airways contracted to CCh (10^{-6} M). There was no difference in the response to isoprenaline under static, compared with oscillatory conditions. n = 6. Mean ± SEM.

Conflict of Interest

The authors declare no conflict of interests.