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TP-receptor activation amplifies airway stretch-activated contractions assessed in perfused intact bovine bronchial segments

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Role of TP-receptor activation in ASM R_{stretch} responses

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Abbreviations:

5-HT – 5-hydroxytryptamine (3-(2-aminoethyl)-1H-indol-5-ol)

AH 6809 – 6-isopropoxy-9-oxoxanthene-2-carboxylic acid

AL 8810 – 9α,15R-dihydroxy-11.beta-fluoro-15-(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-oic acid

ASM – Airway smooth muscle

CCh – Carbachol (2-carbamoyloxyethyl-trimethyl-azanium)

DI – Deep inspiration

FRC – Functional residual capacity

ICI 192605 – 4-(Z)-6-(2-o-Chlorophenyl-4-o-hydroxyphenyl-1,3-dioxan-cis-5-yl)hexenoic acid

Indo - Indomethacin (2-{-1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl}acetic acid)

MAPK – Mitogen-activated protein kinase

MEN 10376 – [Tyr³,D-Trp⁶,⁸,⁹,Lys¹⁰]-NKA(4-10)

NK – Neurokinin

PD 95089 – 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one

R_CCh – CCh-induced bronchial tone

R_stretch,x – contraction evoked by an instantaneous stretch to x cmH₂O

R_U46619 – U46619-induced bronchial tone

TLC – Total lung capacity

Tx – Thromboxane

U46619 – 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid

VSM – Vascular smooth muscle
Recommended section

Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

A deep inspiration (DI) produces bronchodilation in healthy individuals. Conversely, in asthmatics, DIs are less effective in producing bronchodilation, can cause more rapid airway re-narrowing and even bronchoconstriction in moderate to severe asthmatics. Interestingly, the manner by which a DI is able to cause bronchoconstriction via a stretch-activated contraction ($R_{\text{stretch}}$) is thought to correlate positively with airway inflammation. Asthmatic airway inflammation is associated with increased production of thromboxane A$_2$ (TxA$_2$) and subsequent TP-receptor activation, causing the heightened contractility of airway smooth muscle. In this study, we sought to investigate the effect of TxA$_2$ on airway $R_{\text{stretch}}$ using bovine bronchial segments. In brief, these intact bronchial segments (2mm dia.) were dissected, side branches ligated, and the tissues were mounted horizontally in an organ bath. $R_{\text{stretch}}$ was elicited by varying the transmural pressure under isovolumic conditions. Using a pharmacological approach, we showed a reduced $R_{\text{stretch}}$ response in tissues pretreated with indomethacin (Indo), a COX inhibitor; a result mimicked by pretreatment with the TP-selective receptor antagonist ICI 192605, the selective p42/p44 MAPK inhibitor PD 95089, and by airway epithelial denudation. U46619, a TP-receptor agonist elicited enhanced $R_{\text{stretch}}$ responses in a dose-dependent manner. Pretreatment with AH 6809, an EP$_1$/DP-selective receptor antagonist and AL 8810, an FP-selective receptor antagonist had no effect, suggesting EP, DP, and FP-receptor activation are not involved in amplifying ASM $R_{\text{stretch}}$. These data suggest a role for TP-receptor activation and epithelial release of TxA$_2$ in amplifying airway $R_{\text{stretch}}$, thus providing novel insights into mechanisms regulating the DI-induced bronchoconstriction seen in asthmatics.
Introduction

Airways are constantly subjected to mechanical stress due to the inflation and deflation of the lungs. This stress can either produce beneficial (bronchodilatory) responses in healthy individuals or harmful responses (leading to airway hyperresponsiveness) in asthmatics (Maksym, et al., 2005). More specifically, a deep inspiration (DI), clinically measured as a breath taken from functional residual capacity to total lung capacity, produces a bronchodilatory response in ASM of healthy individuals. Conversely, in asthmatics DIs are less effective in producing bronchodilation, can cause more rapid airway re-narrowing, and even bronchoconstriction in moderate to severe asthmatics (Lim, et al., 1987; Gayrard, et al., 1975; Salome, et al., 2003; Jackson, et al., 2004). The mechanisms by which a DI is able to cause bronchoconstriction remain unclear; however, several theories have been postulated explaining how this might occur. Firstly, smooth muscle activation and tension generation may cause an increase in ASM stiffness to the point where it enters a frozen state, in other words, a pro-contractile, high-stiffness, low-hysteresis latch state (An, et al., 2007). Others have reported DI-induced bronchoconstrictions to be a peripheral parenchymal hysteresis-associated event (Lim, et al., 1987). Interestingly, our laboratory has shown, using perfused intact bovine bronchial segments, that airway stretch-activated contractions ($R_{\text{stretch}}$) are dependent upon baseline airway tone and the magnitude of airway stretch. Moreover, we have shown that in intact bovine bronchi, these responses possess non-myogenic characteristics due to the requirement of sensory neuronal input mediated by neurokinin (NK)-A acting through the NK$_2$-receptor (Hernandez, et al., 2008). The inflammation present in asthmatic airways may also amplify airway $R_{\text{stretch}}$ responses. Thus, in this study, we are investigating the role of selected inflammatory mediators in regulating airway $R_{\text{stretch}}$ responses.
Experiments performed in vitro demonstrated that passive sensitization caused $R_{\text{stretch}}$ responses in human airways (Mitchell, et al., 1997), suggesting a role for inflammatory mediators in priming the contractile apparatus to react excessively in the presence of mechanical stress. Among the numerous mediators released in asthmatic airways, prostanoids are both synthesized and released by bouts of airway inflammation as well as by mechanical stress (Allen, et al., 2006; Robinson, et al., 1985). Immunologic challenge of sensitized isolated perfused guinea pig lung, and mechanical stretch of rat lung epithelial cells in vitro, both stimulate prostanoid synthesis and release (Copland, et al., 2006; Robinson, et al., 1984).

In the airway, the major sources of prostanoid synthesis and release include the epithelium, platelets, and alveolar macrophages (Barnes, et al., 1998; Holtzman, 1992). Upon cellular stimulation, prostanoids are synthesized from arachidonic acid liberated from membrane phospholipids by the enzyme phospholipase (PL)-A$_2$ via a p42/44 MAPK-dependent mechanism (Copland, et al., 2006). Arachidonic acid is then converted into PGH$_2$ via cyclooxygenase (COX)-1 and -2. This metabolite is then further converted, by enzyme-dependent reactions, into biologically active prostanoids, namely, prostaglandin (PG)I$_2$ and E$_2$, which produce bronchodilatory (airway protective) features, as well as PGD$_2$, PGF$_{2\alpha}$, and thromboxane (Tx)-A$_2$, which elicit bronchoconstriction (Holtzman, 1992). Among the prostanoids that stimulate ASM, TxA$_2$ has attracted attention as a potential important mediator in the pathophysiology of airway hyperresponsiveness due to the potency of its bronchoconstrictory ability (approx. two orders of magnitude more potent than other prostanoids) (Devillier and Bessard, 1997). Furthermore, clinical studies have demonstrated increased TxA$_2$ concentration in the BAL fluid of asthmatic patients (Barnes, 2001; Lei, et al., 2010; Robinson, et al., 1985). TxA$_2$ elicits its bronchoconstrictory effects by both directly binding to and activating TP-receptors on ASM.
(which signal through the \( G_{\alpha 11} \) family of G proteins) (Kinsella, 2001), as well as by causing prejunctional release of ACh from cholinergic neurons (Allen, et al., 2006; Janssen and Daniel, 1991).

Using a pharmacological approach in intact bovine bronchial segments, as previously described in (Mitchell, et al., 1989), our objective in this study was to determine the effects of the endogenous bronchoconstrictory prostanoids PGD\(_2\), PGF\(_{2\alpha}\), and TxA\(_2\) on \( R_{\text{stretch}} \) responses. In addition, we investigated the possible involvement of the airway epithelium, p42/p44 MAPK, and the TxA\(_2\)-induced prejunctional ACh-release in amplifying these stretch-activated contractions.
Methods

Animals. All experimental procedures were approved by the McMaster University Animal Care Committee (McMaster University, Hamilton, ON, Canada) and conform to the guidelines set by the Canadian Council on Animal Care (Ottawa, ON, Canada). Lower lobes of lung were obtained from cows (200–500 kg) euthanized at a local abattoir and transported to the laboratory in ice-cold modified Krebs buffer solution (116 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.3 mM NaH₂PO₄, 23 mM NaHCO₃, 11mM D-glucose), saturated with 95% oxygen - 5% carbon dioxide to maintain pH at 7.4. Unless indicated otherwise, Krebs buffer did not contain the non-specific cyclooxygenase blocker indomethacin. Upon receipt of the lobes of lung, intact bovine bronchial segments (2 mm diameter, 20 mm length) were carefully dissected free from surrounding parenchyma, excised, and immediately used or stored in modified Krebs solution at 4°C for up to 24 h.

Bronchial segment preparation. For a detailed description of our bronchial segment preparation protocol, please refer to our previous study (Hernandez, et al., 2008). Briefly, following the dissection and excision of the bronchial segment, side branches were tightly ligated. The ligated bronchial segment was then mounted horizontally in a 30 ml Mayflower organ bath (Hugo Sachs Elektronik, March-Hugstetten, Germany) containing warmed modified Krebs buffer solution (37°C) gassed with carbogen (95% O₂ – 5% CO₂). The airway lumen was also filled with warmed modified Krebs solution gassed with carbogen via a jacketed-reservoir, the height of which set the baseline transmural pressure (~5 cmH₂O). This baseline pressure was selected to simulate the transmural pressure found in relaxed airways (Noble, et al., 2007). The connectors at each end of the airway possessed 3-way taps, which could be opened to flush the
airway with modified Krebs solution or closed to make the airway lumen isovolumic. Manual transmural pressure variation was induced under isovolumic conditions by varying the height of perfusate in a column manometer attached distally to the cannulated airway.

Subsequently, we briefly subjected the tissue to an increased transmural pressure load under isovolumic conditions to ensure there were no leaks in the airway. The segment was then left to equilibrate for ~2 hours. During this time, the lumen and adventitia were regularly washed with fresh modified Krebs solution. Following tissue equilibration, transmural pressure was set to ~5 cmH2O. Under isovolumic conditions, tissues were treated with 60 mM KCl (administered extraluminally) and the contractile response (isovolumic increase in transmural pressure) was recorded to test for viability. After washing four times, baseline pressure was reset to ~5 cmH2O.

**Tissue Baths.** To evaluate the effects of mechanical stretch on ASM contraction (measured by transmural pressure generation) in the isolated bronchial segment, we followed the protocol outlined in our previous publication (Hernandez, et al., 2008). In brief, following the tissue viability test, the airway was allowed 20 min of recovery time under isovolumic conditions. Subsequently, electric-field stimulation (EFS) responses were evoked at 5 min intervals until a uniform response was established (after approx. 3-4 repetitions) under isovolumic conditions. EFS was delivered by a train of pulses (60 volts, 2 ms pulse duration, frequency of 20 pulses per second, and 1.5 sec train duration). The airway was then subjected to a transmural pressure pulse of 30 cmH2O, which was maintained for 3 minutes under isovolumic conditions. Transmural pressure was subsequently restored to baseline (~5 cmH2O) and the tissue was allowed 5 min recovery time. To mimic the increased airway tone seen in asthmatic airways, this process was
repeated following pretreatment with 10 nM carbachol (CCh) added to the bath solution to induce submaximal ASM tone under isovolumic conditions. When the agonist-induced tone (R_{CCh}) had reached a plateau (in approx. 10 min), transmural pressure was reset to ~5 cmH₂O before re-assessing airway contractile responses to stretch (R_{stetch,30}) (Fig. 1). The effects of selected contractile agonists on ASM tone was assessed by measuring the rise in transmural pressure in response to increasing concentrations of agonist under isovolumic conditions.

**Pharmacological interventions.** To investigate the pathway involved in amplifying airway stretch-activated contractions, tissues were pretreated extraluminally with a range of different antagonists, whereas the assessment of stretch-activated contractions under control conditions was performed on tissues treated with CCh in a concentration-dependent manner. The possible role for COX was tested by pretreating for 20 min with indomethacin (Indo; 10 µM) (Orehek, et al., 1975), while the roles for EP₁/DP, FP, and TP-receptors were assessed by pretreatment for 20 min with AH 6809 (10 µM) (Coleman, et al., 1987), AL 8810 (10 µM) (Schaafsma, et al., 2005), and ICI 192605 (10 µM) (Janssen and Tazzeo, 2002), respectively (prior to treatment with incremental concentrations of CCh). To further confirm the role for TP-receptors in the amplification of R_{stetch}, tissues were pretreated with the TP-receptor agonist, U46619, in a concentration-dependent manner. To assess any potential cholinergic effect elicited by TP-receptor activation, as previously reported in (Janssen and Daniel, 1991), tissues were pretreated with the muscarinic receptor antagonist, atropine (1 µM; 20 min.) (Russell, 1978), prior to treatment with incremental concentrations of U46619. Lastly, to investigate the role of p42/p44 MAPK in amplifying ASM R_{stetch}, we pretreated tissues with the p42/p44 MAPK inhibitor PD
95089 (10 μM; 20 min.) (Jabbour, et al., 2005), prior to treatment with incremental concentrations of either CCh or U46619.

**Enzyme Immunoassay.** TxA₂ levels were determined in the luminal fluid by measuring its immediate and stable metabolite thromboxane B₂ (TxB₂). A competitive enzyme immunoassay (EIA) for TxB₂ (Cayman Chemical Company, Ann Arbor, MI, USA) was used according to the manufacturer’s instructions (detection limit: 11 pg/ml). Briefly, following the CCh concentration-response protocol outlined in Figure 1, samples were obtained by collecting Krebs buffer solution from the luminal chamber of the tissue bath, which were immediately frozen at -80°C. Control tissues were subjected to increasing concentrations of CCh without transmural pressure pulses used to elicit airway stretch. Prior to beginning the EIA protocol, frozen samples were thawed at room temperature, lyophilized, and solubilized in EIA buffer. The samples were then applied to a 96-well plate pre-coated with mouse anti-rabbit IgG and incubated with TxB₂ antiserum and recovery tracer for 18h. Following incubation, the plates were washed 5x with wash buffer and developed in the dark for 1 hour using Ellman’s reagent. TxB₂ concentrations were determined spectrophotometrically and calculated from the standard curve.

**Epithelial denudation.** To investigate the effect of airway epithelial denudation on \( R_{\text{stretch}} \) responses, the luminal surface of the excised bronchial segment was subjected to mechanical denudation by carefully inserting and retracting a manual probe (3-4 times). Side branches were then ligated with surgical silk and airway segments were mounted onto the Mayflower organ bath as mentioned above.
Histology and staining. Histology procedures followed by staining with Hematoxylin & Eosin (H&E) were used to detect whether the manual probing method was successful in denuding the airway epithelium. Briefly, following excision, a sample of intact and epithelial-denuded airways were submerged in 10% buffered neutral formalin and stored for 48 hours. The tissues were subsequently fixed, embedded in paraffin wax, sliced to a thickness of 6µm with a microtome (Leica, Richmond Hill, ON), placed on a glass slide, and stained with H&E.

Chemicals and solvents. AH 6809, AL 8810, ICI 192605, U46619, and PD 95089 were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). All other pharmacological agents were obtained from Sigma–Aldrich (ON, Canada). The 10 mM stock solutions were prepared in distilled water (atropine, CCh), absolute EtOH (indomethacin), or DMSO (AH 6809, AL 8810, ICI 192605, PD 95089, U46619). Dilutions of these were made in physiological medium; the maximal bath concentration of solvents did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

Statistical Analysis. Stretch-activated contractions ($R_{\text{stretch}}$) were quantified as the difference between the minima and the maxima observed in the transmural pressure recordings following a sudden isovolumic stretch (Fig. 1). All responses were reported as means ± SEM; $n$ refers to the number of animals. TxB$_2$ EIA samples were run in duplicates and TxB$_2$ release was calculated in pg/ml (mean ± SD). Data were fitted to a bell-shaped concentration-response curve which allowed for the measurement of both log EC$_{50}$ and E$_{\text{max}}$. Statistical comparisons between groups were made using the paired or unpaired Student’s $t$-test; $P < 0.05$ was considered statistically significant.
Results

Airway stretch-activated contractions. In resting tissues at a baseline transmural pressure of 5 cmH2O, instantaneously subjecting the tissue to a transmural pressure load of 30 cmH2O led to an instantaneous increase in transmural pressure followed by a more gradual and prolonged isovolumic stress relaxation response (Fig. 1, left). After restoring transmural pressure to baseline, the tissue was challenged with CCh (10 nM) under isovolumic conditions. When this cholinergic tone (RCCh) had stabilized, we reset transmural pressure to 5 cmH2O and allowed 5 minutes for the tissue to re-equilibrate under those new isovolumic conditions before re-assessing the response to a sudden pressure load (30 cmH2O). In contrast to what was seen in the absence of any underlying cholinergic stimulation, the instantaneous spike and transient decrease in transmural pressure (stress relaxation) were now followed by a slowly-developing and prolonged stretch-activated contraction (R_stretch) (Fig. 1, right), the magnitude of which increased with increasing pressure pulse amplitude (Fig. 2A). A more detailed description of this protocol is outlined in our previous study (Hernandez, et al., 2008).

To characterize the mechanisms underlying R_stretch amplification, all subsequent experiments used a standard test pulse of 30 cmH2O (in response to increasing concentrations of either the cholinergic agonist CCh, or the TP-receptor agonist U46619), since the contractile response (R_stretch,30) was maximal at this transmural pressure load (Fig. 2A), and since this mirrors the transmural pressure seen during a deep inspiration to TLC in humans (Scichilone and Togias, 2004).

Relationship between agonist concentration and R_stretch,30. We investigated the dependence of R_stretch,30 upon the degree of excitation produced by agonist-stimulation. There was a substantial
$R_{\text{stretch},30}$ even when tissues were stimulated with CCh at concentrations which evoked little or no direct tone of their own. $R_{\text{stretch},30}$ increased in magnitude with increasing agonist concentrations, reaching a peak at 10 nM CCh, which was sub-maximally effective with respect to evoking direct bronchoconstrictor tone (Fig. 2B). As we have shown previously, higher levels of cholinergic stimulation led to progressively smaller $R_{\text{stretch},30}$ responses.

**Effect of COX inhibition on $R_{\text{stretch},30}$.** To investigate whether arachidonic acid metabolism is involved in $R_{\text{stretch},30}$, we used indomethacin (Indo), a non-selective inhibitor of cyclooxygenase (COX) 1 and 2. All handling of tissues in the control group was done in Indo-free Krebs, while tissues in the treatment group were handled in Krebs containing Indo (10 µM). $R_{\text{stretch},30}$ responses were established following each concentration of a CCh concentration-response protocol. Indo (10 µM) markedly and significantly reduced the $E_{\text{max}}$ of airway $R_{\text{stretch},30}$ responses compared to control ($p<0.05$) (Fig. 3A), but no significant shift in the $EC_{50}$ was observed (Fig. 3A), and there was no effect on $R_{\text{CCh}}$ (Fig. 3B). These data suggest the importance of arachidonic acid metabolites generated by COX in amplifying the magnitude of airway $R_{\text{stretch},30}$ responses without altering $R_{\text{CCh}}$.

**Effect of EP$_1$, DP, FP, and TP-receptor antagonism on $R_{\text{stretch},30}$ and $R_{\text{CCh}}$.** To investigate whether EP, DP, FP, and TP-receptor antagonism would affect $R_{\text{stretch},30}$, we pretreated the tissues with the selective EP$_1$/DP-receptor antagonist AH 6809 (10 µM), the selective FP-receptor antagonist AL 8810 (10 µM), and the selective TP-receptor antagonist ICI 192605 (10 µM) for 20 minutes, then performed a CCh concentration-response protocol, where $R_{\text{stretch},30}$ responses were established following each concentration of CCh. Pretreatment with AH 6809 (10 µM), and
AL 8810 (10 µM) had no effect, whereas ICI 192605 (10 µM) significantly reduced the E_max of $R_{\text{stretch,30}}$ responses compared to control (Fig. 3C). No significant shift in the EC_{50} was observed (Fig. 3C), and $R_{\text{CCh}}$ was not affected (Fig. 3D). These data suggest that TP-receptor activation is involved in amplifying the magnitude of airway $R_{\text{stretch,30}}$ responses without altering $R_{\text{CCh}}$.

**Effect of a TP-receptor agonist (U46619) on $R_{\text{stretch,30}}$ and agonist-induced tone ($R_{\text{U46619}}$).** All tissues used in these experiments were completely handled in Krebs with Indo (10 µM). To investigate the effect of a TP-receptor agonist on $R_{\text{stretch,30}}$ responses, a concentration-response protocol was performed using the selective TP-receptor agonist U46619 (0.1 nM – 1 µM), where $R_{\text{stretch,30}}$ responses were established following each concentration of agonist added. Treatment with U46619 elicited a concentration-dependent increase in $R_{\text{stretch,30}}$ responses with a peak response of 10.90±0.92 cmH$_2$O occurring at a concentration of 0.1 µM (Fig. 4A). This $R_{\text{stretch,30}}$ response occurred with minimal $R_{\text{U46619}}$ (1.12±0.45 cmH$_2$O) (Fig. 4B). These data further strengthen our hypothesis regarding TP-receptor involvement in airway $R_{\text{stretch,30}}$ responses by showing the ability of a selective TP-receptor agonist to elicit $R_{\text{stretch,30}}$ responses in a concentration-dependent manner.

To test for the effect of p42/44 MAPK inhibition on U46619-induced $R_{\text{stretch,30}}$ responses and $R_{\text{U46619}}$, tissues were pretreated with the p42/p44 MAPK inhibitor PD 95089 (10 µM; 20 min.), prior to treatment with incremental concentrations of U46619. Pretreatment with PD 95089 (10 µM) had no effect on the E_max or EC_{50} of the U46619-induced $R_{\text{stretch,30}}$ responses (Fig. 4A) or $R_{\text{U46619}}$ (Fig. 4B).
Effect of airway stretch on TxA2 release. To investigate the effect of airway stretch on the release of TxA2, levels of this arachidonic acid metabolite were determined in the luminal media (Krebs buffer solution) by measuring its immediate and stable metabolite TxB2 using a competitive EIA, as described above. Stretched tissues elicited a significant increase in TxB2 concentration compared to controls (p<0.05) (Fig. 5), suggesting the ability of mechanical stretch to cause the release of TxA2 from the intact bovine bronchial segment.

Effect of epithelial denudation on R_{stretch,30} and R_{CCh}. To determine whether the airway epithelium is a source of the stretch-induced TxA2-release implicated in amplifying the R_{stretch,30} response, we manually denuded the airway epithelium as described above. A CCh concentration-response experiment was then performed, where R_{stretch,30} responses were established following each concentration of CCh. H&E staining of airway tissues confirmed the efficacy of the manual denudation process (described above) in fully removing the airway epithelium, while leaving the lamina propria intact (Fig. 6A i, ii)). Epithelial denudation caused a significant reduction in the E_{max} of R_{stretch,30} responses compared to control (p<0.05), but no difference in EC_{50} was observed (Fig. 6C). R_{CCh} (Fig. 6D) and maximal KCl-induced contraction (R_{KCl}) (Fig. 6B) were not affected.

Role of prejunctional ACh release in TP-receptor activation-induced R_{stretch,30} and R_{U46619}. All tissues used in these experiments were completely handled in Krebs with Indo (10 µM). TP-receptor activation has been shown to contribute to ASM contraction by prejunctionally promoting ACh release from cholinergic neurons (Allen, et al., 2006; Janssen and Daniel, 1991). Thus, to determine whether this phenomenon is implicated in the amplification of R_{stretch}
responses, U46619-induced $R_{\text{stretch,30}}$ responses were generated in the presence of the muscarinic-receptor antagonist atropine (1 µM). $R_{\text{stretch,30}}$ responses were assessed at 0.10 µM U46619, a concentration shown to produce maximal $R_{\text{stretch,30}}$ responses, as described above. Pretreatment with atropine (1 µM) caused no significant changes in maximal U46619 $R_{\text{stretch,30}}$ or $R_{U46619}$, suggesting that the TP-receptor-induced $R_{\text{stretch,30}}$ and $R_{U46619}$ responses are independent of prejunctional ACh release from cholinergic neurons (data not shown).

**Role of p42/44 MAPK in airway $R_{\text{stretch,30}}$ and $R_{\text{CCh}}$.** Stretching airway epithelial cells *in vitro* has been shown to increase prostanoid synthesis and release through a MAPK-dependent mechanism (Copland, et al., 2006). We investigated the possible role of p42/p44 MAPK in the amplification of $R_{\text{stretch,30}}$ by pretreating tissues with the selective p42/p44 MAPK inhibitor PD 95089 (10 µM) for 20 minutes. A CCh concentration-response protocol was then performed, where $R_{\text{stretch,30}}$ responses were established following each concentration of CCh. PD 95089 (10 µM) significantly reduced $R_{\text{stretch,30}}$ $E_{\text{max}}$ responses compared to control (p<0.05), but no difference in $EC_{50}$ was observed (Fig. 7A). $R_{\text{CCh}}$ was not affected (Fig. 7B), suggesting that the amplification of airway $R_{\text{stretch,30}}$ responses is dependent on p42/p44 MAPK activation, while $R_{\text{CCh}}$ is not.
Discussion

In this study, we investigated the effects of the endogenous bronchoconstrictory prostanoids PGD$_2$, PGF$_{2\alpha}$, and TxA$_2$ on R$_{stretch}$ responses using a pharmacological approach in intact bovine bronchial segments. In addition, we provide evidence to suggest the involvement of airway epithelium-derived TxA$_2$ and p42/44 MAPK in the amplification of these R$_{stretch}$ responses.

The concept of stretch inducing a contractile response in ASM is not a novel finding, as it has been previously reported by research groups using both in vitro and ex vivo preparations (Gunst and Russell, 1982; Maksym, et al., 2005; Mitchell, et al., 1997). The novelty of our studies lies in the fact that while previous studies have deemed ASM R$_{stretch}$ to be a myogenic event (Stephens, et al., 1975; Thulesius and Mustafa, 1994), intrinsic to ASM itself, we have demonstrated that this may not be entirely accurate. Using intact bovine bronchial segments, we have shown that airway R$_{stretch}$ are dependent upon contractile machinery priming and the magnitude of airway stretch. Moreover, in intact bovine bronchi, these responses possess non-myogenic characteristics due to the requirement of sensory neuronal input mediated by neurokinin (NK)-A acting through the NK$_{2}$-receptor (Hernandez, et al., 2008).

In Figures 1&2 of this study, we show that contractile machinery priming is required for airway R$_{stretch}$, since these responses occur only when pretreated with submaximally-effective, or even sub-threshold concentrations of CCh. At higher agonist concentrations, the airway segment experiences a lower pre-load volume at the baseline transmural pressure of 5 cmH$_{2}$O due to its higher contractile state, and the stimulation may render the airway too stiff and non-compliant to be able to produce adequate strain following the transmural pressure pulse to generate an R$_{stretch}$ response. Thus, airway smooth muscle contraction per se may not be the main driver for the
R_{stretch} response, because as shown in Figure 2, higher concentrations of CCh, which produced greater bronchial tone, generated smaller R_{stretch} responses. In order to elicit an R_{stretch} response, our data suggest that the airway merely needs to first be ‘primed’ with a submaximal concentration of CCh, and that too much agonist will impede the R_{stretch} response even though bronchial tone is highly elevated. Because of this, our data are best-fit by a bell-shaped curve, showing stimulation at low-concentrations and inhibition at high-concentrations, rather than a sigmoidal curve.

Interestingly, airway inflammation present in asthmatic airways, as shown by ex vivo experiments using passively sensitized human airways (Mitchell, et al., 1997), may add to R_{stretch} responses by the release of stimuli (such as excitatory prostanoids) that prime the contractile apparatus to react excessively in the presence of mechanical stress. Animal studies have demonstrated that these excitatory arachidonic acid metabolites can in fact be synthesized and released by bouts of airway inflammation as well as by mechanical stress (Allen, et al., 2006; Copland, et al., 2006; Robinson, et al., 1984; Robinson, et al., 1985).

In this study, we sought to investigate the possibility that airway R_{stretch} responses may be amplified by the stretch-induced release of excitatory prostanoids. As prostanoids are not typically stored intracellularly after being synthesized, we investigated their role in airway R_{stretch} by inhibiting COX, a key enzyme in the prostanoid synthesis pathway (Holtzman, 1992), present in the airways (Swedin, et al., 2010), and susceptible to inhibition by Indo (Bertolini, et al., 2001). Figures 3A&B show the ability of Indo to significantly reduce the magnitude of R_{stretch,30} without altering R_{CCh}, suggesting a role for excitatory prostanoids in R_{stretch,30} independent of agonist-induced tone generation. In fact, in comparing Fig. 3 to Fig. 4, we see that R_{stretch,30} is of similar magnitude in the presence of CCh versus U46619, even though the former generates a
much larger bronchial tone than the latter. Thus, it is possible that airway $R_{\text{stretch,30}}$ responses possess both tone-dependent and –independent characteristics, where upon reaching a threshold baseline tone, $R_{\text{stretch,30}}$ responses can be significantly augmented with minute increases in concentration of contractile stimuli which are insufficient to alter the airway tone directly.

Upon demonstrating the efficacy of COX-inhibition in significantly reducing airway $R_{\text{stretch,30}}$, we sought to investigate the roles of selected prostanoid receptors (DP, FP, and TP) in amplifying airway $R_{\text{stretch,30}}$ responses. Figures 3C&D show the inability of DP- or FP-receptor antagonism to alter the magnitude of $R_{\text{stretch,30}}$ responses, while TP-receptor antagonism significantly reduced these responses, suggesting the involvement of TP-receptor activation in amplifying $R_{\text{stretch,30}}$. No alteration in $R_{\text{CCCh}}$ was present following treatment with the TP-receptor antagonist (ICI 192605 10 µM), strengthening our hypothesis that $R_{\text{stretch,30}}$ responses may indeed possess both tone-dependent and –independent characteristics. Furthermore, the TP-receptor agonist U46619 generated $R_{\text{stretch,30}}$ responses in a concentration-dependent manner, largely independent of $R_{\text{U46619}}$ and of prejunctional release of ACh from cholinergic neurons, as shown in past studies (Allen, et al., 2006; Janssen and Daniel, 1991). Interestingly, PGD$_2$ and PGF$_{2\alpha}$ have also been shown to exert their effects by binding to the TP-receptor (Dogne, et al., 2002; Lei, et al., 2010), which signal through the $G_{q/11}$ family of G proteins in ASM (Kinsella, 2001), reinforcing the importance of TP-receptor activation in these $R_{\text{stretch,30}}$ responses. In our previous study (Hernandez, et al., 2008), experiments were performed on tissues bathed in Krebs solution containing 10 µM Indo, which would have completely inhibited COX and blocked prostanoid synthesis. Interestingly, $R_{\text{stretch}}$ responses were still elicited, suggesting that these $R_{\text{stretch,30}}$ responses were comprised of the component that is TP receptor-independent. Conversely, in our present study, we performed our control experiments using Indo-free Krebs
solution and observed a significant increase in the magnitude of $R_{\text{stretch},30}$ compared to tissues treated with 10 µM Indo (Fig. 3A), which we attributed to TP-receptor activation, suggesting that TP-receptor activation leads to an amplification of $R_{\text{stretch}}$ responses but is not actually required for $R_{\text{stretch}}$ to occur.

Because of its potency as a bronchoconstrictor (approx. 2 times more potent than other prostanoids) (Devillier and Bessard, 1997), and its increased concentration in the BAL fluid of asthmatic patients (Barnes, et al., 1998; Lei, et al., 2010; Robinson, et al., 1985), TxA2 has attracted attention as a potential important mediator in the pathophysiology of asthma. Here, we showed a significantly increased release of TxB2, the immediate and stable metabolite of TxA2, following transmural pressure loading using a competitive EIA (Fig. 5), demonstrating the ability of mechanical stretch to cause TxA2 release from the airway, as previously shown in cultured rat lung epithelial cells (Copland, et al., 2006).

Moreover, we show the ability of epithelial denudation to significantly reduce $R_{\text{stretch},30}$ to similar levels as that done by COX-inhibition and TP-receptor antagonism (Fig. 6), strengthening previous reports of the epithelium being a major source of prostanoid synthesis and release in response to mechanical stress (Barnes, et al., 1998; Copland, et al., 2006; Holtzman, 1992). Upon cellular stimulation, prostanoids are synthesized from arachidonic acid liberated from membrane phospholipids by the enzyme PLA2 via a MAPK-dependent mechanism (Copland, et al., 2006). Animal studies support that p42/p44 MAPK activation contributes to airway inflammation and hyperresponsiveness (Duan and Wong, 2006), and plays an essential role in stretch-induced prostanoid release from airway epithelium (Copland, et al., 2006). In this study, we demonstrated the ability of a p42/p44 MAPK inhibitor to significantly reduce $R_{\text{stretch},30}$ responses (Fig. 7), showing a role for p42/p44 MAPK in $R_{\text{stretch},30}$ responses. Thus, using our preparation, we
suggest that the p42/p44 MAPK activation occurs at the airway epithelial level prior to TxA2 synthesis and release, as shown in (Copland, et al., 2006).

DI-induced bronchoconstriction is an abnormal phenomenon in humans, since it is only seen in moderate to severe asthmatics. Our bovine bronchial segments were not inflamed nor exhibited spontaneous tone, and did not manifest a stretch-induced contraction until they were pretreated with a contractile agonist (CCh or U46619), used to mimic the increased ASM tone seen in asthmatic airways. Previous studies have also demonstrated an Rstretch in ASM that required pretreatment with a pharmacological agent to prime the contractile apparatus, such as tetraethylammonium chloride, or a cholinergic agonist (Stephens, et al., 1975;Thulesius and Mustafa, 1994). Although others (Ansell, et al., 2009;Gunst, et al., 1990;Noble, et al., 2007) have observed that stretch caused reductions in airway responses to cholinergic stimulation in canine and porcine bronchi, contrasting reports have shown both a lack of stretch-induced relaxation as well as constriction in intact bovine bronchi (Hernandez, et al., 2008;Laprad, et al., 2010).

Although differences in experimental protocols exist between reports, questions have been raised as to whether these differences may be species-related, where bovine ASM is unique in its response to mechanical stretch by behaving more like the asthmatic phenotype (Noble, et al., 2010). These discrepancies may also be attributed to properties of different regions in the airway tree, where Rstretch may be more significant in small resistance airways compared to larger airways.

In conclusion, our data suggest that airway Rstretch may be amplified by bronchoconstrictory prostanoids, namely TxA2, synthesized in a p42/p44 MAPK-dependent manner and released by the airway epithelium in response to stretch. These results highlight an
alternative pathway for potential therapeutic targeting in asthmatic patients where a bronchoconstrictory response to a DI may play a role in airway hyperresponsiveness.
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Authorship Contributions

Participated in research design: Hernandez and Janssen

Conducted experiments: Hernandez

Contributed laboratory equipment: Janssen

Performed data analysis: Hernandez

Wrote or contributed to the writing of the manuscript: Hernandez (minor editing by Janssen)

Other: Janssen acquired funding for the research
References


Footnotes

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Legends for Figures

Fig 1. Representative experimental trace. Pressure recording during the various manipulations used in our experimental protocol; details are given in the Methods, Results, and Discussion. All experiments were performed under isovolumic conditions. The responses to cholinergic stimulation (RCCh) and to pressure pulse stretch (R_stretch) were quantified as illustrated.

Fig 2. Relationship between CCh concentration, pressure pulse magnitude, and bronchial responsiveness to stretch. Agonist was added to the bath 10 min prior to the experimental protocol. (A) Mean magnitudes of R_stretch evoked by transmural pressures of 10-40 cmH2O and CCh concentrations of 1 nM – 0.1 µM (n = 6). (B) Bronchial tone in response to increasing concentrations of CCh (R_CCh) (1 nM – 0.1 µM) (n = 6).

Fig 3. Effect of excitatory prostanoids on R_stretch,30 and RCCh. R_stretch,30 response is represented by a solid line, while R_CCh is represented by a broken line. Effect of COX inhibition on R_stretch,30 (A) and R_CCh (B). All handling of tissues in the control group was done in Indo-free Krebs, while tissues in the treatment group were handled in Krebs pretreated with Indo (10 µM). Effect of selective prostanoid receptor antagonism on R_stretch,30 (C) and R_CCh (D). The EP1/DP-selective receptor antagonist AH 6809 (10 µM), FP-selective receptor antagonist AL 8810 (10 µM), or TP-selective receptor antagonist ICI 192605 (10 µM) was added to the bath 20 min prior to the experimental protocol. R_stretch,30 response and R_CCh were measured at each CCh concentration (1 nM – 0.1 µM) under isovolumic conditions. (n = 6). *, p < 0.05.
Fig 4. Effect of the TP-receptor agonist U46619 on $R_{\text{stretch},30}$ and U46619-induced tone ($R_{U46619}$). $R_{\text{stretch},30}$ response is represented by a solid line, while $R_{U46619}$ is represented by a broken line. (A) $R_{\text{stretch},30}$ response and (B) $R_{U46619}$ were measured at each U46619 concentration (1 nM – 3 µM) under isovolumic conditions. The p42/44 MAPK inhibitor PD 95089 (10 µM) was added to the bath 20 min prior to the experimental protocol. ($n = 6$).

Fig 5. Effect of bronchial stretch on TxB$_2$ release. Experimental details are outlined in the Methods section. Mean values of TxB$_2$ concentrations in the luminal perfusate measured by competitive EIA in unstretched (Control) tissues (open bar), and in stretched tissues (solid bar). ($n = 4$). *, $p < 0.05$. Detection limit: 11pg/ml.

Fig 6. Effect of epithelial denudation on $R_{\text{stretch},30}$, $R_{CCh}$, and maximal response to KCl ($R_{KCl}$). (Ai) & (Aii) H&E-stained bronchial cross-sections demonstrate the efficacy of our epithelial denudation technique (outlined in the Methods section) (100x magnification) ($n = 5$). (C) $R_{\text{stretch},30}$ response and (D) $R_{CCh}$ were measured at each CCh concentration (1 nM – 0.1 µM) under isovolumic conditions ($n = 6$). $R_{\text{stretch},30}$ response is represented by a solid line, while $R_{CCh}$ is represented by a broken line. (B) $R_{KCl}$ (60 mM KCl) was measured 20 min prior to subjecting the tissues to $R_{\text{stretch},30}$ responses in control (solid bar) vs. denuded (open bar) tissues ($n = 6$). *, $p < 0.05$

Fig 7. Effect of p42/44 MAPK inhibition on $R_{\text{stretch},30}$ and $R_{CCh}$. $R_{\text{stretch},30}$ response is represented by a solid line, while $R_{CCh}$ is represented by a broken line. The p42/p44 MAPK-inhibitor PD 95089 (10 µM) was added to the bath 20 min prior to the experimental protocol. (A)
$R_{\text{stretch,30}}$ response and (B) $R_{\text{CCh}}$ were measured at each CCh concentration (1 nM – 0.1 µM) under isovolumic conditions ($n = 6$). *, $p < 0.05$
Figure 4

A

![Graph A showing R_{stretch30} (cmH_2O) vs. U46619 (µM). The graph compares Control (circles) and PD 95089 (squares). Error bars indicate variability.](image)

B

![Graph B showing R_{U46619} (cmH_2O) vs. U46619 (µM). The graph compares Control (circles) and PD 95089 (squares). Error bars indicate variability.](image)
Figure 5

[\text{TxB}_2] (pg/ml)

Unstretched

Stretched

*