Thromboxane Prostanoid Receptor Activation Amplifies Airway Stretch-Activated Constrictions Assessed in Perfused Intact Bovine Bronchial Segments

Jeremy Mark Hernandez and Luke Jeffrey Janssen

Firestone Institute for Respiratory Health, Father Sean O’Sullivan Research Centre, and Department of Medicine, McMaster University, St. Joseph’s Hospital, Hamilton, Ontario, Canada

Received March 30, 2011; accepted July 14, 2011

ABSTRACT

A deep inspiration (DI) produces bronchodilation in healthy individuals. Conversely, in asthmatics, DIs are less effective in producing bronchodilation and can cause more rapid airway narrowing and even bronchoconstriction in moderate to severe asthmatics. It is noteworthy that the manner by which a DI is able to cause bronchoconstriction via a stretch-activated constriction (Rstretch) is thought to correlate positively with airway inflammation. Asthmatic airway inflammation is associated with increased production of thromboxane A2 (TxA2) and subsequent thromboxane prostanoid (TP) receptor activation, causing the heightened contractility of airway smooth muscle. In this study, we sought to investigate the effect of TxA2 on airway Rstretch, by using bovine bronchial segments. In brief, these intact bronchial segments (2 mm in diameter) were dissected, side branches were ligated, and the tissues were mounted horizontally in an organ bath. Rstretch was elicited by varying the transmural pressure under isovolumic conditions. Using a pharmacological approach, we showed a reduced Rstretch response in tissues pretreated with indomethacin, a cyclooxygenase inhibitor, a result mimicked by pretreatment with the TP-selective receptor antagonist 4-[(Z)-6-(2-o-chlorophenyl-4-o-hydroxyphenyl-1,3-dioxan-cis-5-yl)hexenoic acid (ICI 192605) and the selective p42/p44 mitogen-activated protein kinase inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 95089) and by airway epithelial denudation. 9,11-Dideoxy-9α,11α-methanepoxy-prosta-5Z,13E-dien-1-ic acid (U46619), a TP receptor agonist, elicited enhanced Rstretch responses in a dose-dependent manner. Pretreatment with 6-isoproxy-9-o xoaxanthene-2-carboxylic acid (AH 6809), a prostaglandin E (EP) receptor 1/prostaglandin D2 (DP)-selective receptor antagonist, and 9α,15β-dihydroxy-11β-fluoro-15β-(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-ic acid (AL 8810), a prostaglandin F (FP)-selective receptor antagonist, had no effect, suggesting EP, DP, and FP receptor activation is not involved in amplifying airway smooth muscle Rstretch. These data suggest a role for TP receptor activation and epithelial release of TxA2 in amplifying airway Rstretch, thus providing novel insights into mechanisms regulating the DI-induced bronchoconstriction seen in asthmatics.

Introduction

Airways are constantly subjected to mechanical stress caused by the inflation and deflation of the lungs. This stress can either produce beneficial (bronchodilatory) responses in healthy individuals or harmful responses (leading to airway hyper-responsiveness) in asthmatics (Maksym et al., 2005). More specifically, a deep inspiration (DI), clinically measured as an increase in functional residual capacity to total lung capacity, produces a bronchodilatory response in the ASM of healthy individuals. Conversely, in asthmatics DIs are less effective in producing bronchodilation and can cause more rapid airway narrowing and even bronchoconstriction in moderate to severe asthmatics (Gayrard et al., 1975; Lim et al., 1987; Salome et al., 2003; Jackson et al., 2004). The mechanisms by which a DI is able to cause bronchoconstriction are less effectively produced from mechanical stress through the airways. This stress is thought to correlate positively with airway inflammation. Asthmatic airway inflammation is associated with increased production of thromboxane A2 (TxA2) and subsequent thromboxane prostanoid (TP) receptor activation, causing the heightened contractility of airway smooth muscle. In this study, we sought to investigate the effect of TxA2 on airway Rstretch, by using bovine bronchial segments. In brief, these intact bronchial segments (2 mm in diameter) were dissected, side branches were ligated, and the tissues were mounted horizontally in an organ bath. Rstretch was elicited by varying the transmural pressure under isovolumic conditions. Using a pharmacological approach, we showed a reduced Rstretch response in tissues pretreated with indomethacin, a cyclooxygenase inhibitor, a result mimicked by pretreatment with the TP-selective receptor antagonist 4-[(Z)-6-(2-o-chlorophenyl-4-o-hydroxyphenyl-1,3-dioxan-cis-5-yl)hexenoic acid (ICI 192605) and the selective p42/p44 mitogen-activated protein kinase inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 95089) and by airway epithelial denudation. 9,11-Dideoxy-9α,11α-methanepoxy-prosta-5Z,13E-dien-1-ic acid (U46619), a TP receptor agonist, elicited enhanced Rstretch responses in a dose-dependent manner. Pretreatment with 6-isoproxy-9-o xoaxanthene-2-carboxylic acid (AH 6809), a prostaglandin E (EP) receptor 1/prostaglandin D2 (DP)-selective receptor antagonist, and 9α,15β-dihydroxy-11β-fluoro-15β-(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-ic acid (AL 8810), a prostaglandin F (FP)-selective receptor antagonist, had no effect, suggesting EP, DP, and FP receptor activation is not involved in amplifying airway smooth muscle Rstretch. These data suggest a role for TP receptor activation and epithelial release of TxA2 in amplifying airway Rstretch, thus providing novel insights into mechanisms regulating the DI-induced bronchoconstriction seen in asthmatics.
striction remain unclear; however, several theories have been postulated explaining how this might occur. First, 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 procontractile, 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).

It is noteworthy that our laboratory has shown, using perfused intact bovine bronchial segments, that airway stretch-activated contractions ($R_{\text{stretch}}$) depend on baseline airway tone and the magnitude of airway stretch. Moreover, we have shown that in intact bovine bronchi these responses possess nonmyogenic characteristics caused by the requirement of sensory neuronal input mediated by neurokinin (NK)-A acting through the NK$_{\text{A}}$ receptor (Hernandez et al., 2008). The inflammation present in asthmatic airways may also amplify airway $R_{\text{stretch}}$ responses. Thus, in this study, we investigated 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 (Robinson et al., 1985; Allen et al., 2006). 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 (Robinson et al., 1984; Copland et al., 2006).

In the airway, the major sources of prostanoid synthesis and release include the epithelium, platelets, and alveolar macrophages (Holtzman, 1992; Barnes et al., 1998). Upon cellular stimulation, prostanoids are synthesized from arachidonic acid liberated from membrane phospholipids by the enzyme phospholipase A$_2$ via a p42/44 MAPK-dependent mechanism (Copland et al., 2006). Arachidonic acid is then converted into prostaglandin (PG) H$_2$ via cyclooxygenase (COX)-1 and COX-2. This metabolite is then further converted, by enzyme-dependent reactions, into biologically active prostanoids, namely, PGI$_2$ and PGF$_{2\alpha}$, 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 hyper-responsiveness because of the potency of its bronchoconstrictory ability (approximately two orders of magnitude more potent than other prostanoids) (Devillier and Bessard, 1997). Furthermore, clinical studies have demonstrated increased TXA$_2$ concentration in the bronchoalveolar lavage fluid of asthmatic patients (Robinson et al., 1985; Barnes, 2001; Lei et al., 2011). TXA$_2$ elicits its bronchoconstrictory effects by both directly binding to and activating TP receptors on ASM (which signal through the G$_p$Y$_1$ family of G proteins) (Kinsella, 2001), as well as by causing prejunctional release of ACh from cholinergic neurons (Janssen and Daniel, 1991; Allen et al., 2006).

Using a pharmacological approach in intact bovine bronchial segments, as described previously (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, p42/44 MAPK, and the TxA$_2$-induced prejunctional Ach release in amplifying these stretch-activated contractions.
asthmatic airways, this process was repeated after 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_{CC}) had reached a plateau (in approximately 10 min), transmural pressure was reset to ~5 cm H2O before reassessing airway contractile responses to stretch (R_{stretch}) (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 extraluminarily 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 pretreatment with 20 min with Indo (10 µM) (Orehek et al., 1975), whereas the roles for EP1/DP, FP, and TP receptors were assessed by pretreatment for 20 min with 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (AH 6809) (10 µM) (Coleman et al., 1987), 9α,15β-dihydroxy-11β-fluoro-15(2,3-dihydro-1H-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5Z,13E-dien-1-oic acid (AL 8810) (10 µM) (Schaaflma et al., 2005), and 4(Z)-6-2-chlorophenyl-4α-hydroxyphenyl-1,3-dioxan-cis-5-yl)hexenoic acid (ICI 192605) (10 µM) (Janssen and Tazzeo, 2002), respectively (before treatment with incremental concentrations of CCh). To further confirm the role of TP receptors in the amplification of R_{stretch} tissues were pretreated with the TP receptor agonist atropine (1 µM; 20 min) (Russell, 1978), before treatment with incremental concentrations of U46619. Finally, to investigate the role of p42/p44 MAPK in amplifying ASM R_{stretch} we pretreated tissues with the p42/p44 MAPK inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 95089) (10 µM; 20 min) (Jabbour et al., 2005), before 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 TxB2. A competitive enzyme immunoassay (EIA) for TxB2 (Cayman Chemical, Ann Arbor, MI) was used according to the manufacturer’s instructions (detection limit: 11 pg/ml). In brief, following the CCh pretreatment, an aliquot of the tissue luminal fluid was collected in EIA buffer. The samples were then applied to a 96-well plate precoated with mouse anti-rabbit IgG and incubated with TxB2 antiserum and recovery tracer for 18 h. After incubation, the plates were washed five times with wash buffer and developed in the dark for 1 h 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_{stretch} responses, the luminal surface of the excised bronchial segment was subjected to mechanical denudation by carefully inserting and retracting a manual probe (three to four 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 and eosin (H&E) were used to detect whether the manual probing method was successful in denuding the airway epithelium. In brief, after excision, a sample of intact and epithelial-denuded airways were submerged in 10% buffered neutral formalin and stored for 48 h. The tissues were subsequently fixed, embedded in paraffin wax, sliced to a thickness of 6 µm with a microtome (Leica, Richmond Hill, ON, Canada), 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. All other pharmacological agents were obtained from Sigma-Aldrich (Ontario, Canada). The 10 mM stock solutions were prepared in distilled water (atropine, CCh), absolute ethanol (indomethacin), or dimethyl sulphoxide (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_{stretch}) were quantified as the difference between the minima and the maxima observed in the transmural pressure recordings after a sudden isovolumic stretch (Fig. 1). All responses were reported as means ± S.E.M.; n refers to the number of animals. TxB2 EIA samples were run in duplicates, and TxB2 release was calculated in pg/ml (mean ± S.D.). Data were fitted to a bell-shaped concentration-response curve, which allowed for the measurement of both log EC_{50} and E_{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 pull force. This is illustrated in the representative experimental trace (Fig. 1). The pressure recordings during the various manipulations used in our experimental protocol; details are given under Materials and Methods, Results, and Discussion. All experiments were performed under isovolumic conditions. The responses to cholinergic stimulation (R_{CC}) and pressure pulse stretch (R_{stretch}) were quantified as the difference between the minima and the maxima observed in the transmural pressure recordings after a sudden isovolumic stretch (Fig. 1). All responses were reported as means ± S.E.M.; n refers to the number of animals. TxB2 EIA samples were run in duplicates, and TxB2 release was calculated in pg/ml (mean ± S.D.). Data were fitted to a bell-shaped concentration-response curve, which allowed for the measurement of both log EC_{50} and E_{max}. Statistical comparisons between groups were made using the paired or unpaired Student’s t test; P < 0.05 was considered statistically significant.
pressure followed by a more gradual and prolonged isovolumic stress relaxation response (Fig. 1). After restoring transmural pressure to baseline, the tissue was challenged with CCh (10 nM) under isovolumic conditions. When this cholinergic tone (R_{CCh}) had stabilized, we reset transmural pressure to 5 cmH2O and allowed 5 min for the tissue to re-equilibrate under those new isovolumic conditions before reassessing 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 followed by a slowly developing and prolonged stretch-activated contraction (R_{stretch}) (Fig. 1), 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), because the contractile response (R_{stretch,30}) was maximal at this transmural pressure load (Fig. 2A), and this mirrors the transmural pressure seen during a deep inspiration to total lung capacity in humans (Scichilone and Togias, 2004).

**Relationship between Agonist Concentration and R_{stretch,30}** We investigated the dependence of R_{stretch,30} on the degree of excitation produced by agonist stimulation. There was a substantial R_{stretch,30} even when tissues were stimulated with CCh at concentrations that evoked little or no direct tone of their own. R_{stretch,30} increased in magnitude with increasing agonist concentrations, reaching a peak at 10 nM CCh, which was submaximally 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_{stretch,30} responses.

**Effect of COX Inhibition on R_{stretch,30}** To investigate whether arachidonic acid metabolism is involved in R_{stretch,30}, we used Indo, a nonselective inhibitor of COX-1 and COX-2. All handling of tissues in the control group was done in Indo-free Krebs, whereas tissues in the treatment group were handled in Krebs containing Indo (10 μM). R_{stretch,30} responses were established after each concentration of a CCh concentration-response protocol. Indo (10 μM) markedly and significantly reduced the E_{max} of airway R_{stretch,30} responses compared with 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_{CCh} (Fig. 3B). These data suggest the importance of arachidonic acid metabolites generated by COX in amplifying the magnitude of airway R_{stretch,30} responses without altering R_{CCh}.

**Effect of EP_{1}, DP, FP, and TP Receptor Antagonism on R_{stretch,30} and R_{CCh}** To investigate whether EP, DP, FP, and TP receptor antagonism would affect R_{stretch,30} we pre-treated the tissues with the selective EP_{1} 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 min, then performed a CCh concentration-response protocol, where R_{stretch,30} responses were established after 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_{stretch,30} responses compared with control (Fig. 3C). No significant shift in the EC_{50} was observed (Fig. 3C), and R_{CCh} was not affected (Fig. 3D). These data suggest that TP receptor activation is involved in amplifying the magnitude of airway R_{stretch,30} responses without altering R_{CCh}.

**Effect of a TP Receptor Agonist (U46619) on R_{stretch,30} and Agonist-Induced Tone (RU46619)** 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_{stretch,30} responses, a concentration-response protocol was performed using the selective TP receptor agonist U46619 (0.1 nM-1 μM), where R_{stretch,30} responses were established after each concentration of agonist added. Treatment with U46619 elicited a concentration-dependent increase in R_{stretch,30} responses with a peak response of 10.90 ± 0.92 cmH2O occurring at a concentration of 0.1 μM (Fig. 4A). This R_{stretch,30} response occurred with minimal R_{U46619} (1.12 ± 0.45 cmH2O) (Fig. 4B). These data further strengthen our hypothesis regarding TP receptor involvement in airway R_{stretch,30} responses by showing the ability of a selective TP receptor agonist to elicit R_{stretch,30} responses in a concentration-dependent manner.

To test for the effect of p42/44 MAPK inhibition on U46619-induced R_{stretch,30} responses and R_{U46619} tissues were pre-treated with the p42/44 MAPK inhibitor PD 95089 (10 μM; 20 min) before treatment with incremental concentrations of U46619. Pretreatment with PD 95089 (10 μM) had no effect.
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 with 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\text{stretch,30} and R\text{CCh}.

To determine whether the airway epithelium is a source of the stretch-induced TxA₂ release implicated in amplifying the R\text{stretch,30} response, we manually denuded the airway epithelium as described above. A CCh concentration-response experiment was then performed, where R\text{stretch,30} responses were measured at each CCh concentration (1 nM-0.1 μM) under isovolumic conditions (n = 6).
tion caused a significant reduction in the $E_{\text{max}}$ of $R_{\text{stretch,30}}$ responses compared with control ($p < 0.05$), but no difference in EC$_{50}$ was observed (Fig. 6C). $R_{\text{CCh}}$ (Fig. 6D) and maximal KCl-induced contraction ($R_{\text{KCl}}$) (Fig. 6B) were not affected.

**Role of Prejunctional ACh Release in TP Receptor Activation-Induced $R_{\text{stretch,30}}$ and RU46619.** All tissues used in these experiments were completely handled in Krebs with Indo (10$^{-6}$M). TP receptor activation has been shown to contribute to ASM contraction by prejunctionally promoting ACh release from cholinergic neurons (Janssen and Daniel, 1991; Allen et al., 2006). Thus, to determine whether this phenomenon is implicated in the amplification of $R_{\text{stretch,30}}$ responses, U46619-induced $R_{\text{stretch,30}}$ responses were generated in the presence of the muscarinic receptor antagonist atropine (1 $\mu$M). $R_{\text{stretch,30}}$ responses were assessed at 0.10 $\mu$M U46619, a concentration shown to produce maximal $R_{\text{stretch,30}}$ responses, as described above. Pretreatment with atropine (1 $\mu$M) caused no significant changes in maximal U46619 $R_{\text{stretch,30}}$ or $R_{\text{U46619}}$, suggesting that the TP receptor-induced $R_{\text{stretch,30}}$ and $R_{\text{U46619}}$ 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 $\mu$M) for 20 min. A CCh concentration-response protocol was then performed, where $R_{\text{stretch,30}}$ responses were established after each concentration of CCh. PD 95089 (10 $\mu$M) significantly reduced $R_{\text{stretch,30}}$ $E_{\text{max}}$ responses compared with 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 depends on p42/p44 MAPK activation, whereas $R_{\text{CCh}}$ does not.

**Discussion**

In this study, we investigated the effects of the endogenous bronchoconstrictory prostanoids PGD$_2$, PGE$_{2\alpha}$, and TxA$_2$ on $R_{\text{stretch}}$ responses by using a pharmacological approach in intact bovine bronchial segments. In addition, we provide evidence to suggest the involvement of airway epithelium-
derived TxA2 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, because it has been previously reported by research groups using both in vitro and ex vivo preparations (Gunst and Russell, 1982; Mitchell et al., 1997; Maksym et al., 2005). The novelty of our studies lies in the fact that whereas 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 depends on contractile machinery priming and the magnitude of airway stretch. Moreover, in intact bovine bronchi, these responses possess nonmyogenic characteristics caused by the requirement of sensory neuronal input mediated by NK-A acting through the NK2 receptor (Hernandez et al., 2008).

In Figs. 1 and 2, we show that contractile machinery priming is required for airway R_stretch, because these responses occur only when pretreated with submaximally effective, or even subthreshold, concentrations of CCh. At higher agonist concentrations, the airway segment experiences a lower preload volume at the baseline transmural pressure of 5 cmH2O because of its higher contractile state, and the stimulation may render the airway too stiff and noncompliant to be able to produce adequate strain after 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 Fig. 2, higher concentrations of CCh, which produced greater bronchial tone, generated smaller R_stretch responses. 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.

It is noteworthy that 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 mechanical stress (Robinson et al., 1984, 1985; Allen et al., 2006; Copland et al., 2006).

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. Because 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). Figure 3, A and B shows 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 Figs. 3 and 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_stretch,30 responses possess both tone-dependent and -independent characteristics, where upon reaching a threshold baseline tone R_stretch,30 responses can be significantly augmented with minute increases in concentration of contractile stimuli that are insufficient to alter the airway tone directly.

Upon demonstrating the efficacy of COX inhibition in significantly reducing airway R_stretch,30 we sought to investigate the roles of selected prostanoid receptors (DP, FP, and TP) in amplifying airway R_stretch,30 responses. Figure 3, C and D shows the inability of DP or FP receptor antagonism to alter the magnitude of R_stretch,30 responses, whereas TP receptor antagonism significantly reduced these responses, suggesting the involvement of TP receptor activation in amplifying R_stretch,30. No alteration in R_CCh was present after treatment with the TP receptor antagonist (ICI 192605, 10 μM), strengthening our hypothesis that R_stretch,30 responses may indeed possess both tone-dependent and -independent characteristics. Furthermore, the TP receptor agonist U46619 generated R_stretch,30 responses in a concentration-dependent manner, largely independent of R_CCh and prejunctional release of ACh from cholinergic neurons, as shown in past studies (Janssen and Daniel, 1991; Allen et al., 2006). It is noteworthy that PGD2 and PGF2α have also been shown to exert their effects by binding to the TP receptor (Dogné et al., 2002; Lei et al., 2011), which signal through the Gα11 family of G proteins in ASM (Kinsella, 2001), reinforcing the importance of TP receptor activation in these R_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. It is noteworthy that R_stretch,30 responses were still elicited, suggesting that these R_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_stretch,30 compared with 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_stretch,30 responses but is not actually required for R_stretch to occur.

Because of its potency as a bronchoconstrictor (approximately two times more potent than other prostanoids) (Devillier and Bessard, 1997), and its increased concentration in the bronchoalveolar lavage fluid of asthmatic patients (Robinson et al., 1985; Barnes et al., 1998; Lei et al., 2011), 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, after transmural pressure loading by using a competitive EIA (Fig. 5), demonstrating the ability of mechanical stretch to cause TxA2 release from the airway, as shown previously in cultured rat lung epithelial cells (Copland et al., 2006). Moreover, we show the ability of epithelial denudation to significantly reduce R_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 (Holtzman, 1992; Barnes et al., 1998; Copland et al., 2006). Upon cellular stimulation, prostanoids are synthesized from arachidonic acid liberated from membrane phospholipids by the enzyme phospholipase A₂ via a MAPK-dependent mechanism (Copland et al., 2006). Animal studies support that p42/p44 MAPK activation contributes to airway inflammation and hyper-responsiveness (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 iRstretch,30 responses (Fig. 7), showing a role for p42/p44 MAPK in iRstretch,30 responses. Thus, using our preparation, we suggest that the p42/p44 MAPK activation occurs at the airway epithelial level before TxA₂ synthesis and release, as shown previously (Copland et al., 2006).

DI-induced bronchoconstriction is an abnormal phenomenon in humans, because it is only seen in moderate to severe asthmatics. Our bovine bronchial segments were not inflamed, did not exhibit 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 tetroxathylammonium chloride, or a cholinergic agonist (Stephens et al., 1975; Thulesius and Mustafa, 1994). Although others (Gunst et al., 1990; Noble et al., 2007; Ansell et al., 2009) 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 with larger airways.

In conclusion, our data suggest that airway Rstretch may be amplified by bronchoconstrictory prostanoitds, 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 hyper-responsiveness.

Acknowledgments

We thank Tracy Tazzeo for the bovine lungs used in this study; Dr. Gerard Cox for the Mayflower tissue bath apparatus; Marg Cote for help performing the TxB₂ enzyme immunoassay; and Lindsay DoHarris for technical assistance with the histology and staining protocols.

Authorship Contributions

Participated in research design: Hernandez and Janssen.

Conducted experiments: Hernandez.

Contributed new reagents or analytic tools: Janssen.

Performed data analysis: Hernandez.

Wrote or contributed to the writing of the manuscript: Hernandez.

References


Address correspondence to: Luke Jeffrey Janssen, Firestone Institute for Respiratory Health, St. Joseph’s Hospital, Room L-314, 50 Charlton Avenue, East Hamilton, ON, Canada L8N 4A6. E-mail: janssenl@mcmaster.ca