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

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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 renarrowing 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 contraction ($R_{stretch}$) is thought to correlate positively with airway inflammation. Asthmatic airway inflammation is associated with increased production of thromboxane A_2 (TxA_2) and subsequent thromboxane prostanoid (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 $R_{stretch}$ 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. $R_{stretch}$ was elicited by varying the transmural pressure under isovolumic conditions. Using a pharmacological approach, we showed a reduced $R_{stretch}$ 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)-4*H*-1-benzopyran-4-one (PD 95089) and by airway epithelial denudation. 9,11-Dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid (U46619), a TP receptor agonist, elicited enhanced $R_{stretch}$ responses in a dose-dependent manner. Pretreatment with 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (AH 6809), a prostaglandin E (EP) receptor 1/prostaglandin D2 (DP)-selective receptor antagonist, and 9α,15*R*-dihydroxy-11.β-fluoro-15-(2,3-dihydro-1*H*-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5*Z*,13*E*-dien-1-oic 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 $R_{stretch}$. These data suggest a role for TP receptor activation and epithelial release of TxA_2 in amplifying airway $R_{stretch}$, 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 a breath taken from 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 renarrowing 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 bronchocon-

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ABBREVIATIONS: DI, deep inspiration; ACh, acetylcholine; AH 6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; AL 8810, 9α,15*R*-dihydroxy-11.β-fluoro-15-(2,3-dihydro-1*H*-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5*Z*,13*E*-dien-1-oic acid; ASM, airway smooth muscle; CCh, carbachol (2-carbamoyloxyethyl-trimethyl-azanium); COX, cyclooxygenase; DP, prostaglandin D2; EIA, enzyme immunoassay; EP, prostaglandin E; FP, prostaglandin F; H&E, hematoxylin and eosin; 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-1*H*-indol-3-yl}acetic acid]; MAPK, mitogen-activated protein kinase; NK, neurokinin; PD 95089, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; PG, prostaglandin; R_{CCh}, CCh-induced bronchial tone; $R_{stretch}$, contraction evoked by an instantaneous stretch to cmH_2O; TP, thromboxane prostanoid; Tx, thromboxane; U46619, 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid; R_{U46619}, U46619-induced bronchial tone.

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 ($R_{stretch}$) 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_2 receptor (Hernandez et al., 2008). The inflammation present in asthmatic airways may also amplify airway $R_{stretch}$ responses. Thus, in this study, we investigated the role of selected inflammatory mediators in regulating airway $R_{stretch}$ responses.

Experiments performed in vitro demonstrated that passive sensitization caused $R_{stretch}$ 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 PGE_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 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 $G_{q/11}$ 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 $R_{stretch}$ 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.

Materials and Methods

Animals. All experimental procedures were approved by the McMaster University Animal Care Committee (McMaster University, Hamilton, ON, Canada) and conformed 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 $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11 mM D-glucose), saturated with 95% oxygen-5% carbon dioxide to maintain pH at 7.4. Unless indicated otherwise, Krebs buffer did not contain the nonspecific cyclooxygenase blocker indomethacin (Indo). 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). In brief, after the dissection and excision of the bronchial segment, side branches were tightly ligated. The ligated bronchial segment was then mounted horizontally in 30 ml of Mayflower organ bath (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) containing warmed modified Krebs buffer solution (37°C) gassed with carbogen (95% O_2-5% CO_2). 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 cmH_2O). 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 three-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 h. During this time, the lumen and adventitia were regularly washed with fresh modified Krebs' solution. After tissue equilibration, transmural pressure was set to ~5 cmH_2O. 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 cmH_2O.

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, after the tissue viability test, the airway was allowed 20 min of recovery time under isovolumic conditions. Subsequently, electric-field stimulation responses were evoked at 5-min intervals until a uniform response was established (after approximately three to four repetitions) under isovolumic conditions. electric-field stimulation was delivered by a train of pulses (60 V, 2-ms pulse duration, frequency of 20 pulses per second, and 1.5-s train duration). The airway was then subjected to a transmural pressure pulse of 30 cmH_2O, which was maintained for 3 min under isovolumic conditions. Transmural pressure was subsequently restored to baseline (~5 cmH_2O), and the tissue was allowed 5-min recovery time. To mimic the increased airway tone seen in

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_{CCh}) had reached a plateau (in approximately 10 min), transmural pressure was reset to ~5 cmH_2O before reassessing airway contractile responses to stretch ($R_{stretch,30}$) (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 pretreatment for 20 min with Indo (10 μM) (Orehek et al., 1975), whereas the roles for EP_1/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,15*R*-dihydroxy-11.β-fluoro-15-(2,3-dihydro-1*H*-inden-2-yl)-16,17,18,19,20-pentanor-prosta-5*Z*,13*E*-dien-1-oic acid (AL 8810) (10 μM) (Schaafsma et al., 2005), and 4-(*Z*)-6-(2-*o*-chlorophenyl-4-*o*-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 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid (U46619), in a concentration-dependent manner. To assess any potential cholinergic effect elicited by TP receptor activation, as reported previously (Janssen and Daniel, 1991), tissues were pretreated with the muscarinic receptor antagonist 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)-4*H*-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. TxA_2 levels were determined in the luminal fluid by measuring its immediate and stable metabolite TxB_2. A competitive enzyme immunoassay (EIA) for TxB_2 (Cayman Chemical, Ann Arbor, MI) was used according to the manufacturer's instructions (detection limit: 11 pg/ml). In brief, following the CCh concentration-response protocol outlined in Fig. 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. Before 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 precoated with mouse anti-rabbit IgG and incubated with TxB_2 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. TxB_2 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 sulfoxide (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. TxB_2 EIA samples were run in duplicates, and TxB_2 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 cmH_2O, instantaneously subjecting the tissue to a transmural pressure load of 30 cmH_2O led to an instantaneous increase in transmural

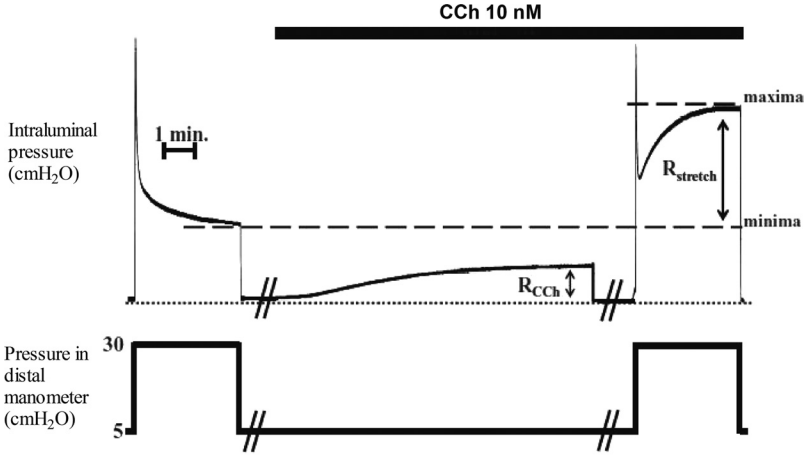

Fig. 1. Representative experimental trace. Pressure recording during the various manipulations used in our experimental protocol; details are given under *Materials and Methods*, *Results*, and *Discussion*. All experiments were performed under isovolumic conditions. The responses to cholinergic stimulation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

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 cmH_2O 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 cmH_2O). 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 cmH_2O (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.

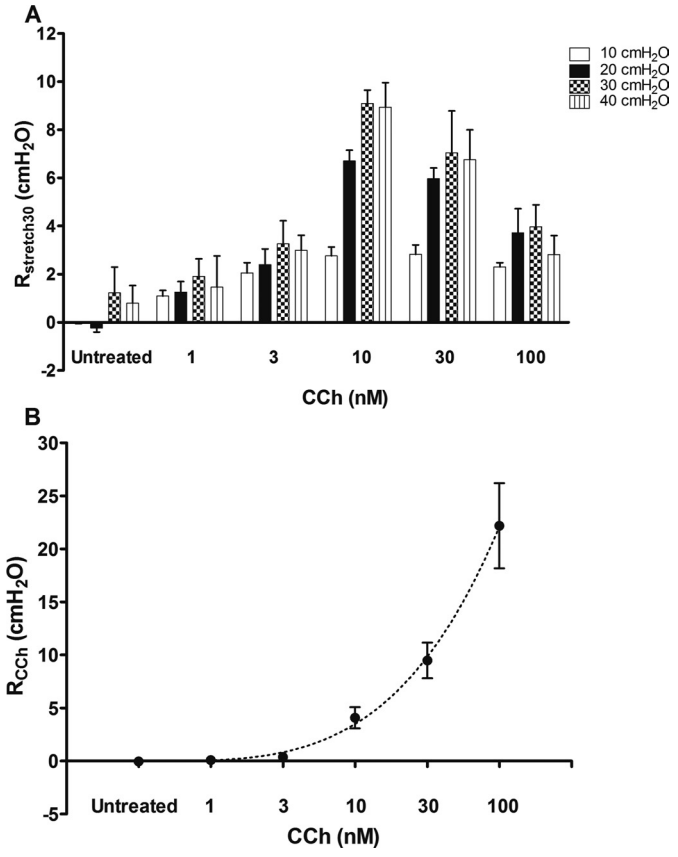

Fig. 2. Relationship between CCh concentration, pressure pulse magnitude, and bronchial responsiveness to stretch. Agonist was added to the bath 10 min before the experimental protocol. A, mean magnitudes of $R_{stretch}$ evoked by transmural pressures of 10 to 40 cmH_2O and CCh concentrations of 1 nM to 0.1 μM ($n = 6$). B, bronchial tone in response to increasing concentrations of CCh (R_{CCh}) (1 nM-0.1 μM) ($n = 6$).

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 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 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 (R_{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 $R_{stretch,30}$ 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 cmH_2O occurring at a concentration of 0.1 μM (Fig. 4A). This $R_{stretch,30}$ response occurred with minimal R_{U46619} (1.12 ± 0.45 cmH_2O) (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 pretreated with the p42/p44 MAPK inhibitor PD 95089 (10 μM; 20 min) before treatment with incremental concentrations of U46619. Pretreatment with PD 95089 (10 μM) had no effect

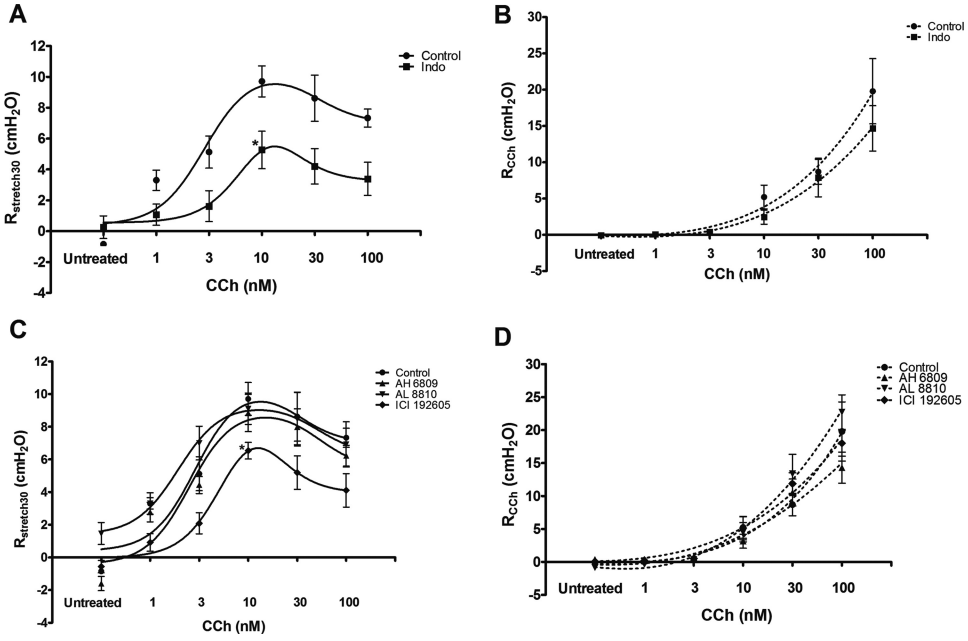

Fig. 3. Effect of excitatory prostanoids on $R_{stretch,30}$ and R_{CCh}. $R_{stretch,30}$ response is represented by solid lines, and R_{CCh} is represented by broken lines. A and B, effect of COX inhibition on $R_{stretch,30}$ (A) and R_{CCh} (B). All handling of tissues in the control group was done in Indo-free Krebs, whereas tissues in the treatment group were handled in Krebs pretreated with Indo (10 μM). C and D, effect of selective prostanoid receptor antagonism on $R_{stretch,30}$ (C) and R_{CCh} (D). The EP_1/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 before the experimental protocol. $R_{stretch,30}$ response and R_{CCh} were measured at each CCh concentration (1 nM-0.1 μM) under isovolumic conditions ($n = 6$). *, $p < 0.05$.

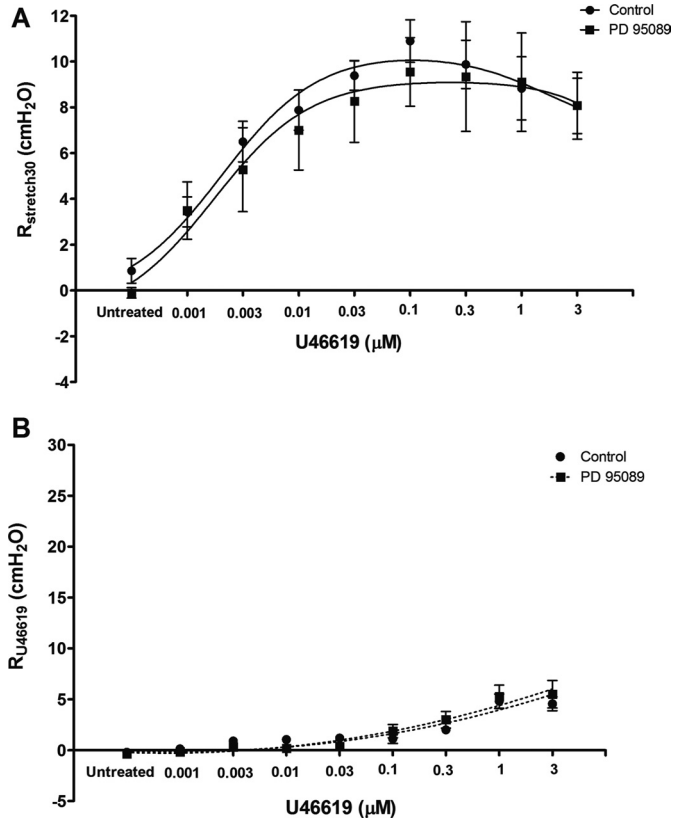

Fig. 4. Effect of the TP receptor agonist U46619 on $R_{stretch,30}$ and U46619-induced tone (R_{U46619}). $R_{stretch,30}$ response is represented by solid lines, and R_{U46619} is represented by broken lines. $R_{stretch,30}$ response (A) and R_{U46619} (B) were measured at each U46619 concentration (1 nM-3 μM) under isovolumic conditions. The p42/44 MAPK inhibitor PD 95089 (10 μM) was added to the bath 20 min before the experimental protocol ($n = 6$).

on the E_{max} or EC_{50} of the U46619-induced $R_{stretch,30}$ responses (Fig. 4A) or R_{U46619} (Fig. 4B).

Effect of Airway Stretch on TxA_2 Release. To investigate the effect of airway stretch on the release of TxA_2, levels of this arachidonic acid metabolite were determined in the luminal media (Krebs buffer solution) by measuring its immediate and stable metabolite TxB_2 using a competitive EIA, as described above. Stretched tissues elicited a significant increase in TxB_2 concentration compared with controls ($p < 0.05$) (Fig. 5), suggesting the ability of mechanical stretch to cause the release of TxA_2 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 TxA_2 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 after 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). Epithelial denuda-

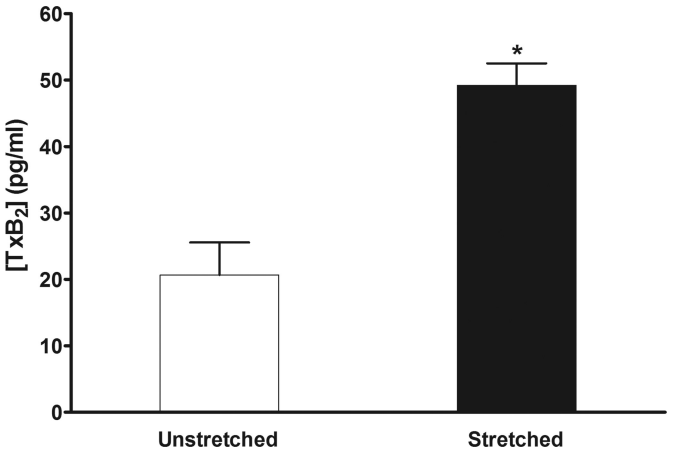

Fig. 5. Effect of bronchial stretch on TxB_2 release. Experimental details are outlined under *Materials and Methods*. Mean values of TxB_2 concentrations in the luminal perfusate measured by competitive EIA in unstretched (control) tissues (open bar) and stretched tissues (solid bar) are shown ($n = 4$). *, $p < 0.05$. Detection limit was 11 pg/ml.

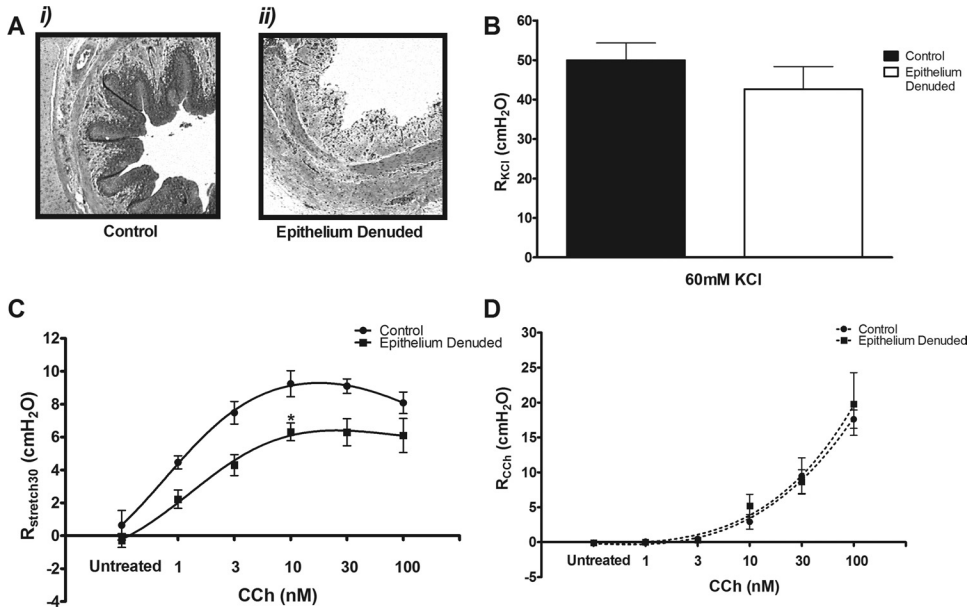

Fig. 6. Effect of epithelial denudation on $R_{stretch,30}$, R_{CCh}, and maximal response to KCl (R_{KCl}). A, H&E-stained bronchial cross-sections demonstrate the efficacy of our epithelial denudation technique (outlined under *Materials and Methods*) (100× magnification) ($n = 5$). B, R_{KCl} (60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

tion caused a significant reduction in the E_{max} of $R_{stretch,30}$ responses compared with 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 (Janssen and Daniel, 1991; Allen et al., 2006). Thus, to determine whether this phenomenon is implicated in the amplification of $R_{stretch}$ responses, U46619-induced $R_{stretch,30}$ responses were generated in the presence of the muscarinic receptor antagonist atropine (1 μM). $R_{stretch,30}$ responses were assessed at 0.10 μM U46619, a concentration shown to produce maximal $R_{stretch,30}$ responses, as described above. Pretreatment with atropine (1 μM) caused no significant changes in maximal U46619 $R_{stretch,30}$ or R_{U46619}, suggesting that the TP receptor-induced $R_{stretch,30}$ and R_{U46619} responses are independent of prejunctional ACh release from cholinergic neurons (data not shown).

Role of p42/44 MAPK in Airway $R_{stretch,30}$ and R_{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 $R_{stretch,30}$ by pretreating tissues with the selective p42/p44 MAPK inhibitor PD 95089 (10 μM) for 20 min. A CCh concentration-response protocol was then performed, where $R_{stretch,30}$ responses were established after each concentration of CCh. PD 95089 (10 μM) significantly reduced $R_{stretch,30}$ E_{max} responses compared with control ($p < 0.05$), but no difference in EC_{50} was observed (Fig. 7A). R_{CCh} was not affected (Fig. 7B), suggesting that the amplification of airway $R_{stretch,30}$ responses depends on p42/p44 MAPK activation, whereas R_{CCh} does not.

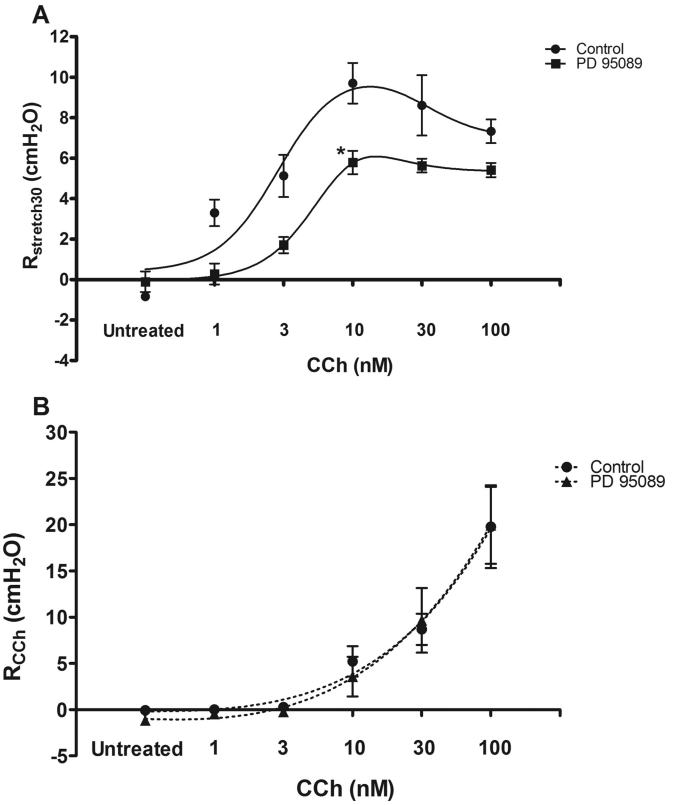

Fig. 7. Effect of p42/44 MAPK inhibition on $R_{stretch,30}$ and R_{CCh}. $R_{stretch,30}$ response is represented by solid lines, and R_{CCh} is represented by broken lines. The p42/p44 MAPK inhibitor PD 95089 (10 μM) was added to the bath 20 min before the experimental protocol. $R_{stretch,30}$ response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

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 by 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, 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 NK_2 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 cmH_2O 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_{U46619} 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 PGD_2 and $PGF_{2\alpha}$ 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 $G_{q/11}$ 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 $R_{stretch}$ 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 $R_{stretch}$ 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), TxA_2 has attracted attention as a potential important mediator in the pathophysiology of asthma. Here, we showed a significantly increased release of TxB_2, the immediate and stable metabolite of TxA_2, after transmural pressure loading by using a competitive EIA (Fig. 5), demonstrating the ability of mechanical stretch to cause TxA_2 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 A_2 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 $R_{stretch,30}$ responses (Fig. 7), showing a role for p42/p44 MAPK in $R_{stretch,30}$ responses. Thus, using our preparation, we suggest that the p42/p44 MAPK activation occurs at the airway epithelial level before TxA_2 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 $R_{stretch}$ 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 (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 $R_{stretch}$ may be more significant in small resistance airways compared with larger airways.

In conclusion, our data suggest that airway $R_{stretch}$ may be amplified by bronchoconstrictory prostanoids, namely TxA_2, 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.

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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.

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