Cholinergic Receptor and Cyclic Stretch-Mediated Inflammatory Gene Expression in Intact ASM

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We tested the hypothesis that cholinergic stimulation and cyclic stretch regulate inflammatory gene expression in intact airway smooth muscle by measuring mRNA expression in bovine tracheal smooth muscle using limited microarray analysis and RT-PCR. Carbachol (1 μ M) induced significant increases in the expression of cyclooxygenase (COX)-1, COX-2, IL-8, and plasminogen activator, urokinase type (PLAU) to levels ranging from 1.3- to 3.1-fold of control. Sinusoidal length oscillation at an amplitude of 10% muscle length and a frequency of 1 Hz induced significant increases in the expression of CCL-2, COX-2, IL-1 β , and IL-6 to levels ranging from 12- to 206-fold of control. Decreasing the oscillatory amplitude by 50% did not significantly change inflammatory gene expression. In contrast, decreasing the oscillatory frequency by 50% significantly attenuated inflammatory gene expression by 76–93%. Nifedipine (1 μ M) had an insignificant effect on carbachol-induced gene expression, but significantly inhibited sinusoidal length oscillation-induced inflammatory gene expression by 40–78%. Correlation analysis revealed two groups of genes with differential responses to sinusoidal length oscillation. The highly responsive group included COX-2, IL-6, and IL-8, which exhibited 45- to 364-fold increases in gene expression in response to sinusoidal length oscillation. The moderately responsive group included CCL2 and PLAU, which exhibited 13- to 19-fold increases in gene expression in response to sinusoidal oscillation. These findings suggest that cyclic stretch regulates inflammatory gene expression in intact airway smooth muscle in an amplitude- and frequency-dependent manner by modulating the activity of L-type voltage-gated calcium channels.

Keywords: airway smooth muscle; COX-2; IL-6; IL-8; nifedipine

Excessive airway smooth muscle contraction in response to airway inflammation is an important cause of asthma. In addition, recent studies indicate that airway smooth muscle cells are capable of secreting chemokines, cytokines, and other inflammatory mediators such as RANTES and monocyte chemoattractant proteins (1). Chemokines secreted by airway smooth muscle cells in response to inflammatory stimuli may lead to the recruitment of inflammatory cells to the vicinity of airway smooth muscle cells, further increasing the inflammatory stimuli to airway smooth muscle and thereby amplifying airway inflammation. Published studies on the secretory function of airway smooth muscle cells have been derived almost exclusively from experiments performed on cultured airway smooth muscle cells that are stimulated by a mixture of inflammatory cytokines. Smooth muscle cells are capable of undergoing significant phenotypic

modulation ranging from a highly synthetic/proliferative phenotype to a highly contractile/differentiated phenotype depending on the chemical and mechanical environment (2). Therefore, it is not known to what extent inflammatory genes are expressed in intact differentiated airway smooth muscle. Furthermore, airway smooth muscle cells are innervated by cholinergic nerves *in vivo*, and normally function in an oscillatory mechanical environment during respiratory cycles. It is also not known how cholinergic stimulation and cyclic stretch regulate inflammatory gene expression in intact airway smooth muscle.

In a recent study, we found that cholinergic stimulation and sinusoidal length oscillation interactively regulate gene expression of myosin isoforms and α -SM actin in intact bovine tracheal smooth muscle (3). This finding has led us to hypothesize that cholinergic stimulation and cyclic stretch regulate inflammatory gene expression in intact airway smooth muscle. In this study, we tested this hypothesis by first performing a limited microarray analysis of gene expression in bovine tracheal smooth muscle to identify inflammatory genes regulated by carbachol or sinusoidal length oscillation. We then performed semiquantitative RT-PCR experiments to measure gene expression in bovine tracheal smooth muscle stimulated by carbachol and sinusoidal length oscillation at various oscillatory amplitudes and frequencies. Activation of L-type voltage-gated calcium channels has been found to mediate depolarization-induced cyclic AMP-response element (CRE)-dependent gene expression in vascular smooth muscle (4), and CRE is known to be involved in the regulation of inflammatory gene expression (5). Therefore, we investigated the effect of nifedipine, an antagonist of L-type calcium channels on cholinergic receptor-mediated and cyclic stretch-induced inflammation gene expression in bovine tracheal smooth muscle.

MATERIALS AND METHODS

Tissue Preparation

Bovine tracheae were collected from a slaughterhouse and transported to the laboratory in ice-cold physiological salt solution (PSS) of the following composition (in mM): 140.1 NaCl, 4.7 KCl, 1.2 Na₂HPO₄, 2.0 MOPS (pH 7.4), 0.02 Na₂EDTA, 1.2 MgSO₄, 1.6 CaCl₂, and 5.6 D-glucose. The smooth muscle layer together with adventitia and mucosa was excised from a tracheal segment and placed in a petri dish of cold PSS. The mucosal and adventitial layers were dissected away from the smooth muscle layer under a microscope. Smooth muscle strips, ~ 4 mm in width, were prepared along the direction of muscle fibers. The ends of each muscle strip were tied with surgical silk. For length oscillation experiments, one end of each muscle strip was tied to a stainless steel hook, which was connected to the arm of a lever for length oscillation while the other end was secured at a clamp connected to a length manipulator.

Experimental Protocols

The procedures for tissue equilibration and length manipulation have been described previously (3, 6, 7). Briefly, a computer program controls the sending of voltages to the length input port of the lever system at regular time intervals, thereby inducing sinusoidal oscillation of the lever arm and the muscle strip attached to the lever arm. For length oscillation experiments, muscle strips were first stretched to 12 g and

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then allowed to equilibrate for 1 h in PSS bubbled with air at 37°C. The muscle strips were then activated for 3 min with K-PSS, a solution similar to PSS in composition except that 104.95 mM NaCl was substituted by an equimolar concentration of KCl. Viable muscle strips were then allowed to relax in PSS for 15 min, and stretched to 12 g every 15 min during another hour of equilibration in PSS. Muscle strips were adjusted to reference length (L_0) for contraction by releasing the muscle strips quickly to 2.5 g, and then stimulated by K-PSS for 10 min. Muscle strips were then allowed to relax in PSS for 15 min, and the muscle length was measured using a caliper (resolution = 0.1 mm). Four muscle strips were set up for each experiment, and each muscle strip was treated by one of the following four experimental protocols: (A) no drug, no oscillation; (B) carbachol, no oscillation; (C) no drug, oscillation; (D) carbachol, oscillation. In protocol "A," muscle strips were incubated in PSS without any mechanical stretch. In protocol "B," muscle strips were incubated in carbachol-containing PSS without any mechanical stretch. In protocol "C," muscle strips were incubated in PSS with sinusoidal length oscillation. In protocol "D," muscle strips were incubated in carbachol-containing PSS with sinusoidal length oscillation. Solutions were refreshed every hour, and the total duration of treatment was 4 h. Muscle strips were stretched sinusoidally using a computer-controlled Dual Mode Lever System (Model 300 B; Aurora Scientific Inc., Aurora, ON, Canada) and DMC/DMA Version 3.1 software.

RNA Extraction and Reverse Transcription

After completion of each experiment, the muscle strips were homogenized in 300 μ l of lysis solution with 1% 2-mercaptoethanol (GenElute Mammalian Total RNA Kit; Sigma, St. Louis, MO). Tissue homogenate was then digested with proteinase K solution (Sigma) for 10 min at 55°C, followed by centrifugation at 10,000 rpm for 10 min at 23°C. RNA extraction was performed on the supernatant using mini columns according to the manufacturer's instructions. The concentration of the eluted RNA was determined by ultraviolet spectrophotometry (DU-64 spectrophotometer; Beckman, Fullerton, CA). To remove any contaminating DNA, the RNA was treated with DNase I (RNase-free DNase I; Sigma) following the manufacturer's protocol. The ImProm-II Reverse transcription system (Promega, Madison, WI) was used for reverse transcription of the RNA. RNA (0.25 μ g) was first combined with 0.5 μ g of random primers, and water was added to a final volume of 5 μ l. The RNA/primer mixture was next incubated in a 70°C heat block for 5 min, followed by 10 min on ice. The following reagents (Promega) were then added to the tube for reverse transcription, giving a final volume of 20 μ l: 4 μ l of ImProm-II 5 $\times$ reaction buffer, 2.8 μ l of 25 mM $MgCl_2$ (final concentration 3.5 mM), 1 μ l of 10 mM dNTP mix (final concentration 0.5 mM), 20 units of recombinant RNasin, 1 μ l of ImProm-II reverse transcriptase, 1 μ l of 25 mM DTT (final concentration 1.25 mM), 2 μ g of acetylated-BSA, and 2.7 μ l of nuclease-free water. The reverse transcription reaction was run in a GeneAmp PCR system 2,400 thermocycler (Applied Biosystems, Foster City, CA) using the following conditions as recommended by Promega: 5 min at 25°C, then 60 min at 42°C, and finally 15 min at 70°C to inactivate the reverse transcriptase enzyme.

Microarray Analysis

Custom microarrays for 402 experimental genes and 78 "house-keeping" genes were designed and fabricated by the Microarray Core Facility at Boston University Medical Center. Briefly, 50 mer probes for the microarray were designed by first identifying 50-bp sequences within the target cDNA sequence that were unique within the *Bos taurus* BTGI genome database and then filtering these to minimize predicted RNA secondary structure and to select sequences with a predicted annealing temperature of $84 \pm 6^\circ\text{C}$. These steps were performed with the program ArrayOligoSelector (8). Among the five best candidate-probe sequences, the probe that hybridized to the most 3' region of the target was selected. For 54 genes that we wished to include on the microarray, an orthologous *Bos taurus* sequence had not been identified. For these genes we searched the *Bos taurus* BTGI genome database for the most homologous sequence and used this in the probe selection process. Probes were then synthesized and spotted in duplicate. RNA samples extracted from bovine muscle strips (unstimulated, and stimulated by carbachol or sinusoidal oscillation) were analyzed. After reverse

transcription, each RNA sample was split and labeled with Cy3 or Cy5. One Cy3- and one Cy5-labeled sample was hybridized to each array in a randomized-block design. Thus, each RNA sample was analyzed on two independent arrays. The resulting hybridization intensities were scaled to a mean value of 1,000 and duplicate spots were averaged. Genes that exhibited differential expression as a result of oscillation or drug treatment were identified using a mixed-model ANOVA that included effects for oscillation, drug treatment, and the labeling dye. The P value threshold used for evidence of differential expression was determined by calculating the False Discovery Rate to control for multiple-hypothesis testing (9). In the microarray "heat map" the color of each cell reflects the fold-change of the observed hybridization intensity relative to the average hybridization intensity across all samples. Saturated green cells represent a 2-fold decrease in hybridization intensity, while saturated red cells represent a 2-fold increase. The raw dataset is available from the Gene Expression Omnibus under accession no. GSE4287.

Real-Time PCR

Bovine-specific TaqMan PCR reagents were designed for the inflammatory genes identified as altered in expression by microarray analysis as well as for an endogenous control, bovine β -actin (Table 1) using the computer software Primer Express version 1.5 (Applied Biosystems). TaqMan probes were labeled at the 5' end with the fluorescent reporter dye 6FAM (6-carboxyfluorescein) and at the 3' end with a nonfluorescent quencher dye (MGBNFQ) (Applied Biosystems). These reagents were used to determine mRNA transcription levels in three various treatments of bovine airway smooth muscle (oscillation/no carbachol, no oscillation/carbachol, and oscillation/carbachol) in each experiment relative to that of the control treatment (no oscillation/no carbachol). The reaction volume was 50 μ l, containing 5 μ l of a 1:10 dilution of BASM cDNA derived from the reverse transcription procedure, 900 nM final concentration of each of the appropriate forward and reverse primers, 250 nM final concentration of the corresponding probe, 25 μ l of TaqMan universal PCR master mix with AmpErase UNG (Applied Biosystems), and 5 μ l of nuclease-free water. Real-time TaqMan PCR was performed using the ABI Prism 7,300 Sequence Detection System and 96-well optical plates (Applied Biosystems). Reaction conditions (identical to the manufacturer's default) were as follows: Stage 1, 1 cycle of 50°C for 2 min; Stage 2, 1 cycle of 95°C for 10 min; and Stage 3, 40 cycles of 95°C for 15 s and 60°C for 1 min. Target and endogenous control gene (β -actin) reactions were performed in separate wells and each reaction was run in triplicate. Differential expression (relative quantification) was determined as compared with the control treatment (no oscillation/no drug) with the ABI 7,300 SDS software (Applied Biosystems) using the comparative C_T method according to the manufacturer's manual. Briefly, all target gene TaqMan PCR reaction results (average cycling threshold or C_T values) were normalized to those of the endogenous control gene bovine β -actin to correct for any differences in efficiency of the reverse transcription reactions; this value is termed ΔC_T . Next, for each target gene, the normalized average C_T (ΔC_T) values for the control samples (no oscillation, no carbachol) were subtracted from the ΔC_T values for the experimentally treated samples, to give the value $\Delta\Delta C_T$. This data was converted to relative quantification values (relative to the control sample) for each target gene in each experimentally treated sample using the formula $2^{-\Delta\Delta C_T}$.

Statistical Analysis of RT-PCR Data

Data are presented as means $\pm$ SE; n represents the number of animals. Student's t test was used for the comparison of two means ($P < 0.05$ considered significant). Correlation between expression levels of two genes was analyzed by correlation analysis and linear regression using the least-square method.

RESULTS

Microarray Analysis

Figure 1 shows the heat map of the microarray data for the genes that exhibited the most significant changes (mixed-model ANOVA P value < 0.001 , which corresponds to a False Discovery Rate < 0.05). These genes included inflammatory cytokines

TABLE 1. PRIMER AND TAQMAN SEQUENCES FOR REAL-TIME PCR

Gene Name (Accession Number)	Forward Primer Reverse Primer	Taqman Probe
β -Actin (AY141970)	TCATCACCATCGGCAATGAG GGATGTCGACGTACACTTCA	CATTACGAAACTACC
CCL2 (NM_174006)	CCATGCAGAGGCTGATGAAT CTTGCCCAGGATGGTCTTGA	CACCAGCAGCAAGTG
COX-1 (AF004943)	TCAAGGCACCAACCTCATGTT GGACCCATCTTGCCAGAAGTT	ACCCATCAGTCTCTCA
COX-2 (AF031698)	CCAGAGCTGCTTTTCAACCAA AGCGTGTTAAACTCAGCAGCAA	TCCAGTACCAGAACCGT
IL-1 β (NM_174093)	AAGGCTCTCCACCTCCTCTCA ACCCAAGGCCACAGGAATCT	AACACCACTTCTCGGTTT
IL-6 (NM_173923)	TGTGAAAGCAGCAAGGAGACA TGATCAAGCAAATCGCCTGAT	ATAAGCTGAATCTTCC
IL-8 (NM_173925)	GAGAGTGGGCCACACTGTGAA GGCAGACCTCGTTTCCATTG	CAGAAATCATTGTTAAGCTT
PLAU (NM_174147)	GAGAGCCTTGCTCACCACAAT CACACTGGCCTCTGCTGGTA	ATCTTCAGCAAGGCAAT
TNF- α (NM_173966)	GTTCTCACCCACACCATCAG AGGGCTCTTGATGGCAGACA	CATTGCAGTCTCCTACC

Definition of abbreviations: CCL2, chemokine C-C motif ligand 2; COX, cyclooxygenase; PLAU, plasminogen activator, urokinase type.

Taqman probes were labeled with the fluorescent dye 6FAM at the 5' end and a nonfluorescent quencher (MGBNFQ) at the 3' end (Applied Biosystems).

(IL-1 β , IL-6), chemokines (CCL2, IL-8), enzymes involved in inflammation (cyclooxygenase [COX]-2, plasminogen activator, urokinase type [PLAU]), and other signaling proteins. In Figure 1, rectangles under each experimental protocol represent results from the 10 hybridization intensities obtained for each experimental treatment group. Comparison of the untreated samples (Group 1: -/-) with the carbachol treated samples (Group 2: -/+) suggests that cholinergic receptor activation induces inflammatory gene expression. Comparison of the untreated samples (Group 1: -/-) with the samples that have been subjected to oscillation (Group 3: +/-) suggested that sinusoidal length oscillation also induces expression of these genes.

Effect of Carbachol and Length Oscillation on Gene Expression

Based on the microarray data, we performed semiquantitative RT-Real-Time PCR experiments to determine the effects of cholinergic receptor activation and sinusoidal length oscillation on inflammation gene expression. We focused on the six inflammatory genes (CCL2, COX-2, IL-1 β , IL-6, IL-8, and PLAU) as indicated by the microarray data. We added COX-1 as a control for COX-2 and added TNF- α because of its close relationship to IL-1 β . We used the β -actin gene as an endogenous control for carrying out the $\Delta\Delta C_T$ method. The primer and Taqman sequences for these genes are shown in Table 1.

Figure 2 shows the effects of cholinergic stimulation and sinusoidal length oscillation on inflammatory gene expression. As shown in Figure 2A, carbachol (1 μ M) induced significant changes in the gene expression of IL-8, COX-2, PLAU, and COX-1 to 2.2-, 3.1-, 1.7-, and 1.3-fold of the values of control (unstimulated, unstretched tissues), respectively. Figure 2B shows the effect of sinusoidal length oscillation at an amplitude of 10% muscle length, and frequency of 1 Hz on gene expression in unstimulated and carbachol-stimulated tissues. As shown in Figure 2B (*solid bars*), sinusoidal length oscillation of unstimulated tissues induced significant increases in the expression of IL-6, COX-2, CCL2, and IL-1 β to 206-, 45.3-, 12.5-, and 11.8-fold of control, respectively, and significant decrease in COX-1 expression by 10%. As shown in Figure 2B (*shaded bars*), sinusoidal length oscillation of carbachol-activated tissues induced

significant increases in the expression of IL-6, IL-8, COX-2, PLAU, and CCL2 to 364-, 99-, 103-, 16.2-, and 18.9-fold of control, respectively. Comparison of Figures 2A and 2B suggested that the combined effect of cholinergic stimulation and sinusoidal length oscillation was greater than the sum of individual effects in stimulating inflammatory gene expression in bovine tracheal smooth muscle.

Amplitude and Frequency Dependencies of Cyclic Stretch-Induced Gene Expression

Figure 3 shows the effect of decreasing either amplitude or frequency of sinusoidal length oscillation on inflammatory gene expression in unstimulated tissues. As shown in Figure 3A, decreasing oscillatory amplitude from 10% to 5% muscle length at the same oscillatory frequency of 1 Hz did not significantly change inflammatory gene expression in unstimulated tissues. In contrast, as shown in Figure 3B, decreasing oscillatory frequency from 1 to 0.5 Hz at the same oscillatory amplitude of 10% muscle length significantly decreased gene expression of IL-6, COX-2, CCL2, and IL-1 β by 93, 82, 77, and 76%, respectively, in unstimulated tissues.

Figure 4 shows the effect of decreasing either amplitude or frequency of sinusoidal length oscillation on gene expression in carbachol-stimulated tissues. As shown in Figure 4A, decreasing oscillatory amplitude from 10% to 5% muscle length at the same oscillation frequency of 1 Hz had insignificant effect on the expression of most genes except PLAU, which decreased significantly by 63%, in carbachol-stimulated tissues. In contrast, decreasing oscillatory frequency from 1 to 0.5 Hz at the same oscillatory amplitude of 10% muscle length significantly decreased gene expression of IL-6, IL-8, COX-2, PLAU, and CCL2 by 95, 87, 81, 64, and 83%, respectively, in carbachol-stimulated tissues (Figure 4B).

Effect of Nifedipine on Inflammatory Gene Expression

Figure 5 shows the effect of the calcium channel antagonist, nifedipine (1 μ M), on gene expression stimulated by 1 μ M carbachol and/or sinusoidal length oscillation. As shown in Figure 5A, nifedipine had insignificant effect on carbachol-stimulated inflammatory

Figure 1. Microarray data showing the ten genes that exhibited the most reproducible changes (mixed-model ANOVA P value < 0.001) in response to 1 μ M carbachol and/or sinusoidal length oscillation at amplitude of 10% muscle length and frequency of 1 Hz. The three experimental conditions are indicated by the absence (–) or presence (+) of carbachol or sinusoidal oscillation. There are five samples per experimental condition, each of which has been hybridized to two arrays for a total of 10 gene expression measurements per experimental condition. For each sample the two gene expression measurements are in adjacent columns. The color of each cell reflects the fold-change of the observed hybridization intensity for that gene relative to the average hybridization intensity across all samples. Saturated green cells represent a 2-fold decrease in hybridization intensity, while saturated red cells represent a 2-fold increase. CCL2, chemokine C-C motif ligand 2; COX-2, cyclooxygenase-2; IGFBP3, insulin-like growth factor-binding protein 3; NR2F1, nuclear receptor subfamily 2, group F, member 1; ITGA6, integrin α 6; PKC, protein kinase C; PLAU, plasminogen activator, urokinase type.

gene expression. In contrast, as shown in Figure 5B, nifedipine (1 μ M) significantly attenuated the increases in IL-6 and CCL2 expression in response to sinusoidal length oscillation in unstimulated tissues by 62 and 40%, respectively. Similarly, as shown in Figure 5C, nifedipine significantly attenuated the increases in the gene expression of IL-6, IL-8, COX-2, PLAU, and CCL2 in carbachol-stimulated tissues by 78, 64, 67, 71, and 72%, respectively.

Correlation Analysis

Examination of the gene expression patterns in Figures 2–4 suggested two distinct groups of genes exhibiting noticeably different degrees of upregulation in response to sinusoidal length oscillation. The highly responsive group included IL-6, IL-8, and COX-2, which exhibited 45- to 364-fold increases in gene expression in response to sinusoidal length oscillation (Figure 2B). The moderately responsive group included PLAU and CCL2, which exhibited only 13- to 19-fold increases in gene expression in response to sinusoidal oscillation (Figure 2B). We further analyzed the expression levels of these two groups of data to determine correlation among the expression levels of genes within each group. Using COX-2 expression as a reference, Figure 6 plots the expression levels of COX-2, IL-6, IL-8, PLAU, and CCL2 against the expression level of COX-2 expression in

Figure 2. Effect of (A) 1 μ M carbachol and (B) sinusoidal length oscillation at amplitude of 10% muscle length and frequency of 1 Hz with or without 1 μ M carbachol on gene expression of IL-6, IL-8, COX-2, PLAU, CCL2, IL-1 β , TNF- α , and COX-1. Gene expression level is normalized by the level expressed in unstretched, unstimulated tissues (control; solid bars). Striped bars, oscillation + carbachol. Bars and vertical lines represent means $\pm$ SE ($n = 3$). Asterisks indicate significant differences from control.

all experiments in this study. COX-2 expression was chosen as the reference because COX-2 expression exhibited the most consistent changes in this study. As shown in Figure 6, the expression of IL-6 and IL-8 correlated with the expression of COX-2, with correlation coefficients of 0.96 and 0.98, respectively. Furthermore, COX-2, IL-6, and IL-8 appeared to cluster together as a group (Figure 6, closed symbols), indicating similar degrees of up-expression for these three genes. The expression of PLAU and CCL2 also correlated with the expression of COX-2 with correlation coefficients of 0.76 and 0.96, respectively. PLAU and CCL2 appeared to cluster as another group (Figure 6, open symbols), and the slopes for these two genes were lower than those for COX-2, IL-6, and IL-8, indicating lesser degree of upregulation for PLAU and CCL2.

DISCUSSION

In this study, we found that cholinergic receptor activation and sinusoidal length oscillation both significantly induced the expression of inflammatory genes in bovine tracheal smooth muscle, although the effect of sinusoidal length oscillation was greater (Figure 2). Although the number of samples is relatively

Figure 3. (A) Amplitude and (B) frequency dependencies of gene expression induced by sinusoidal length oscillation in unstimulated tissues. In A, unstimulated tissues were stretched sinusoidally at the same frequency of 1 Hz with amplitudes of either 5% (open bars) or 10% muscle length (filled bars). In B, unstimulated tissues were stretched sinusoidally at either 0.5 Hz (open bars) or 1 Hz (filled bars) at the same amplitude of 10% muscle length. Bars and vertical lines represent means $\pm$ SE ($n = 3$). Gene expression level is normalized by the level expressed in unstretched, unstimulated tissues (control). Asterisks indicate significant differences in gene expression between the two experimental groups in each panel.

Figure 4. (A) Amplitude and (B) frequency dependencies of gene expression induced by sinusoidal length oscillation in 1 μ M carbachol-stimulated tissues. In A, carbachol-stimulated tissues were stretched sinusoidally at the same frequency of 1 Hz with amplitudes of either 5% (open bars) or 10% muscle length (filled bars). In B, carbachol-stimulated tissues were stretched sinusoidally at either 0.5 Hz (open bars) or 1 Hz (filled bars) at the same amplitude of 10% muscle length. Gene expression level is normalized by the level expressed in unstretched, unstimulated tissues (control). Bars and vertical lines represent means $\pm$ SE ($n = 3$). Asterisks indicate significant differences in gene expression between the two experimental groups in each panel.

small for the real-time PCR experiments in this study, many significant changes in inflammatory gene expression were detected possibly because many changes were relatively large and consistent. For example, four genes were found to be significantly upregulated by carbachol, and five genes were found to be significantly upregulated or downregulated by sinusoidal oscillation. The observed changes in inflammatory gene expression in this study are likely to be an underestimate of the total number of genes that were sensitive to cholinergic receptor activation and cyclic stretch. Carbachol (1 μ M) induced significant increases in the expression of COX-1, COX-2, IL-8, and PLAU to levels ranging from 1.3-fold of control (COX-1) to 3.1-fold of control (COX-2). In contrast, sinusoidal length oscillation at amplitude of 10% muscle length, and frequency of 1 Hz induced significant increases in the expression of CCL-2, COX-2, IL-1 β , and IL-6 to levels ranging from 12-fold of control (IL-1 β) to 206-fold of control (IL-6), but downregulated COX-1 expression by $\sim$ 10%. IL-6 is a pleiotropic cytokine that exhibits both proinflammatory

and anti-inflammatory properties in asthma (1). CCL2 and IL-8 are chemokines important in the recruitment of inflammatory cells in asthma and chronic obstructive pulmonary disease (10, 11). COX-2 is the inducible cyclooxygenase isoform that is upregulated in allergic inflammation and asthma (12). PLAU is an activator of the plasmin system that degrades extracellular matrix during airway remodeling (13). Therefore, these genes are potentially relevant to airway inflammation and remodeling. Barnes (14) identified IL-10, IL-12, IL-18, IL-23, and IFN- γ as anti-inflammatory cytokines in the airways. We searched our microarray data for the expression of genes encoding these cytokines, but found that neither carbachol nor cyclic stretch significantly altered the expression of IL-12 (accession number U14416), IL-18 (accession number NM_174091), and IFN- γ (accession number NM_174086). The false discovery rate values for the effects of cholinergic receptor activation and sinusoidal length oscillation were near 1 for all of these genes, indicating the lack of statistical significance. Therefore, cholinergic receptor activation and cyclic

Figure 5. Effect of 1 μ M nifedipine on gene expression stimulated by (A) 1 μ M carbachol, (B) sinusoidal length oscillation at 10% muscle length and 1 Hz, and (C) sinusoidal length oscillation together with carbachol. Untreated (filled bars) and nifedipine-treated (open bars) tissues were otherwise stimulated with identical experimental conditions. Gene expression level is normalized by the level expressed in unstretched, unstimulated tissues (control). Bars and vertical lines represent means $\pm$ SE ($n = 3$). Asterisks indicate significant differences in gene expression between untreated and nifedipine-treated tissues.

strain appeared to stimulate inflammatory cytokine expression without significant effects on anti-inflammatory cytokine expression.

Airway smooth muscle cells are innervated by cholinergic nerves, and normally function in a mechanically active environ-

Figure 6. Correlation analysis of the expression of CCL2 (open triangles), COX-2 (filled circles), IL-6 (filled triangles), IL-8 (filled squares), and PLA2 (open circles) in relation to COX-2 expression. Data points were taken from Figures 2–5. Lines represent linear regression fits to individual data sets.

ment during breathing cycles. Recent findings suggested that airway smooth muscle cells are capable of secreting inflammatory cytokines, thereby participating in the inflammatory process in airways (1). The observed cholinergic receptor-mediated and cyclic stretch-induced increases in the expression of CCL2, COX-2, IL-6, IL-8, and PLA2 in this study are consistent with this suggestion. Inflammatory stimulus such as IL-1 β , TNF- α , and IFN- γ have been shown to induce COX-2 expression and secretion of CCL2, IL-6, and IL-8 in cultured human airway smooth muscle cells (15–19). Bradykinin and cyclic mechanical stretch have been shown to induce IL-8 expression in cultured human airway smooth muscle cells (20, 21). Cholinergic regulation of airway smooth muscle secretory function has been suggested but has not been demonstrated in airway smooth muscle cells, probably because the expression of cholinergic receptors is rapidly downregulated under cell culture conditions (22). To our knowledge, this is the first report of cholinergic receptor- and cyclic stretch-induced expression of inflammatory gene expression in intact airway smooth muscle.

Cyclic stretch and cholinergic receptor activation both stimulated inflammatory gene expression in bovine tracheal smooth muscle. However, the following lines of evidence suggest that cholinergic receptor activation is unlikely to be the underlying mechanism of cyclic stretch-induced inflammatory gene expression. First, the effect of cyclic stretch was much greater than the effect of carbachol on inflammatory gene expression (Figure 2). Second, the combined effect of cyclic stretch and carbachol together was greater than the effect of cyclic stretch alone (Figure 2B). Third, nifedipine significantly inhibited cyclic stretch-induced gene expression, but had insignificant effect on carbachol-mediated gene expression (Figure 5). We have previously found that the same oscillatory parameters induced significant changes in α -SM actin expression in bovine tracheal smooth muscle (3), but the extent of change was much lower than those observed in this study. The effects of cholinergic receptor activation and sinusoidal stimulation on inflammatory gene expression appeared to be synergistic. For example, when tissues were stimulated by both carbachol and sinusoidal length oscillation, IL-6 expression reached 364-fold of control level, whereas IL-6 expression levels were only 206-fold and 5.7-fold of control level when the two stimuli were applied individually (Figure 2).

Amplitude and frequency are the two parameters that define the waveform of sinusoidal length oscillation. The oscillatory amplitude and frequency (10% muscle length, 1 Hz) used in

this study are similar to those for inducing the expression of inflammatory cytokine in cultured airway smooth muscle cells (21). We found that decreasing the oscillatory amplitude by 50% from 10% to 5% muscle length did not significantly change inflammatory gene expression in both unstimulated and carbachol-stimulated bovine tracheal smooth muscle (Figures 3 and 4). In contrast, decreasing the oscillatory frequency by 50% from 1 Hz to 0.5 Hz significantly attenuated inflammatory gene expression by 76–93%. To our knowledge, this is the first report of differential effects of oscillatory amplitude and frequency on inflammatory gene expression in airway smooth muscle. This finding is intriguing in terms of the normal amplitude and frequency of breathing cycles. Fredberg and coworkers (23) studied the effect of sinusoidal stretching on contractile force in bovine tracheal smooth muscle, and estimated that normal tidal breathing would correspond to 4% of muscle length, and a sigh would correspond to 12% of muscle length. The observed relative insensitivity of inflammatory gene expression to oscillatory amplitude between 5% and 10% muscle length at 1 Hz frequency suggests that normal tidal lung inflations at relatively high frequency (1 Hz) could induce inflammatory gene expression in airway smooth muscle. The observed dramatic attenuation of inflammatory gene expression at 0.5 Hz frequency while maintaining oscillatory amplitude at 10% muscle length suggests that high tidal volumes do not necessarily induce inflammatory gene expression in airway smooth muscle as long as the ventilation frequency is relatively low (0.5 Hz). Deep inspirations are known to have strong bronchodilatory and bronchoprotective effects on the normal airways, but the response is lost in individuals with asthma (24). However, prolonged lung ventilation at high tidal volumes can lead to lung inflammation and injury (25). Results from this study suggested that oscillatory frequency is an important determinant of whether a given oscillatory amplitude would induce inflammatory gene expression in airway smooth muscle. This suggestion is consistent with the findings of Conrad and colleagues (26) that reducing ventilation frequency attenuated ventilator-induced lung injury in animals. Recent studies suggest that ventilation with low lung volumes can also induce lung injury due to lung collapse and repetitive opening and closing of alveoli (27, 28). Identification of the molecular mechanisms of cyclic stretch-induced lung cell activation is important for developing novel ventilatory and pharmacologic strategies for preventing the deleterious effects of mechanical ventilation (29). Results from this study suggest that amplitude and frequency are interactive variables in eliciting the inflammatory response of lung cells in response to cyclic stretch.

The calcium channel antagonist, nifedipine (1 μ M), had an insignificant effect on carbachol-induced inflammatory gene expression in bovine tracheal smooth muscle (Figure 5A). This finding was consistent with the findings of Hirota and coworkers (30) that 1 μ M nifedipine had a relatively small effect on carbachol-induced airway constriction. In contrast, 1 μ M nifedipine significantly attenuated sinusoidal length oscillation-induced inflammatory gene expression by 40–78% in both unstimulated and carbachol-stimulated tissues (Figures 5B and 5C). This finding suggests that L-type voltage-gated calcium channels are involved in cyclic stretch-induced inflammatory gene expression in airway smooth muscle. This suggestion was consistent with the findings of Wamhoff and colleagues (4) that L-type voltage-gated calcium channels were involved in depolarization-induced activation of the CRE-dependent promoter via CaM kinase-dependent phosphorylation of the CRE-binding protein in vascular smooth muscle. CRE has been identified at the promoter region of the following genes: COX-2, IL-6, IL-8, and PLA2 (31–34). To our knowledge, it remains unknown whether CRE is present at the CCL2 promoter. However, 8-bromo-cAMP has

been found to revert the effects of COX-2 inhibition on CCL2 expression induced by TNF- α and IL-1 α in hepatic stellate cells (35), suggesting that cAMP mediates the effect of prostaglandins on CCL2 expression. In contrast, 8-bromo-cAMP has been found to decrease CCL2 expression induced by IL-1 β in human airway smooth muscle cells (36), suggesting that cAMP antagonizes CCL2 expression. Therefore, cAMP appears to regulate CCL2 expression although the exact role of cAMP remains unclear. These findings together suggest that CRE may be a common element in cyclic strain-stimulated expression of COX-2, IL-6, IL-8, PLA2, and possibly CCL2. Intracellular [Ca²⁺] increase has been shown to play a permissive role in stretch-induced IL-6 secretion from endothelial cells (37). Relatively little has been published on stretch-induced activation of L-type voltage-gated calcium channels in airway smooth muscle. However, several studies suggested that mechanical stretch-induced activation of L-type voltage-gated calcium channels possibly by activating integrin receptors could be an important mechanism of myogenic vascular smooth muscle contraction (38–41). To our knowledge, this is the first report of nifedipine's inhibitory effect on cyclic stretch-induced inflammatory gene expression in airway smooth muscle. It is noteworthy that inhibition of L-type voltage-gated calcium channels appears to have no effect on cyclic stretch-induced Erk1/2 MAPK activation in alveolar epithelial cells (42). Therefore, airway smooth muscle and alveolar epithelial cells appear to use different mechanosensitive signaling mechanisms in response to cyclic stretch, and yet both cell types are capable of producing inflammatory cytokines.

Correlation analysis of the data in this study revealed strong correlation among the expression of CCL2, COX-2, IL-6, IL-8, and PLA2 (Figure 6). In addition, the analysis revealed two distinct groups of genes exhibiting noticeably different degrees of upregulation in response to sinusoidal length oscillation (Figure 6). The highly responsive group included COX-2, IL-6, and IL-8, which exhibited 45- to 364-fold increases in gene expression in response to sinusoidal length oscillation. The moderately responsive group included CCL2 and PLA2, which exhibited 13- to 19-fold increases in gene expression in response to sinusoidal oscillation. However, both groups appeared to be sensitive to nifedipine with similar levels of attenuation (Figure 5). We speculate that activation of L-type voltage-gated calcium channels may be a primary step in cyclic stretch-induced stimulation the expression of CCL2, COX-2, IL-6, IL-8, and PLA2, but additional downstream mechanisms modulate the expression of CCL2 and PLA2 in intact airway smooth muscle. Cyclooxygenases are rate-limiting enzymes in prostaglandin synthesis (43). Therefore, the observed upregulation of COX-2 expression raised the possibility that prostaglandins may be involved in the upregulation of cytokine expression by cyclic stretch. It is noteworthy that genetic COX-2 deficiency has been found to augment inflammation in airways in one study (44), but have insignificant effect on T helper type 2 airway response to allergen challenge in another study (45). Therefore, the role of COX-2 in airway inflammation is not completely understood. We hope to investigate the role of COX-2 in cyclic stretch-induced inflammatory cytokine expression in future experiments. Recently, cyclic stretch has been found to stimulate inflammatory gene expression in various smooth muscle types. For example, cyclic stretch has been found to stimulate the expression of IL-6 in vascular smooth muscle cells (46), and IL-8 in uterine smooth muscle cells (47). Recently, Adam and coworkers (48) reported that cyclic stretch stimulated more than 2-fold increases in the expression of only 20 out of 11,731 genes ($\sim 0.17\%$) in bladder smooth muscle cells. These findings together with ours suggest that mechanosensitive regulation of inflammatory gene expression may be a general property of all smooth muscle cell types.

We speculate that smooth muscle cells may serve as biomechanical sensors in the wall of internal organs for modulating the inflammatory process in response to changes in the mechanical environment. In summary, findings from this study suggest that cyclic stretch regulates inflammatory gene expression in intact airway smooth muscle in an amplitude- and frequency-dependent manner by modulating the activity of L-type voltage-gated calcium channels.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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