Chronic Inflation of Ferrets Lungs with CPAP Reduces Airway Smooth Muscle Contractility In vivo and In vitro

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Running Head: Chronic CPAP Reduces Airway Responsiveness

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ABSTRACT

The mechanical stress imposed on the lungs during breathing is an important modulator of airway responsiveness in vivo. Our recent study demonstrated that continuous positive airway pressure (CPAP) applied to the lungs of non-anesthetized, tracheotomized rabbits for four days decreased lower respiratory system responsiveness to challenge with ACh. In addition, airway segments excised from the lungs of these animals and studied in vitro exhibited reduced contractility. However, the mechanism for this reduction in contractility was not determined. The stress-induced decrease in airway responsiveness could have resulted from alterations in the excitation-contraction coupling mechanisms of the smooth muscle cells, or it might reflect changes in the structure and/or composition of the airway wall tissues. In the present study, we assessed the effect of prolonged chronic stress of the lungs in vivo on airway smooth muscle force generation, MLC-phosphorylation and airway wall structure. To enhance the potential development of stress-induced structural changes, we applied mechanical stress for a prolonged period of time of 2 - 3 weeks. Our results demonstrate a direct connection between the decreased airway responsiveness caused by chronic mechanical stress of the lungs in vivo and a persistent decrease in contractile protein activation in the airway smooth muscle isolated from those lungs. The chronic stress also caused an increase in airway size, but no detectable changes in the composition of the airway wall.

Key words: mechanical stress; MLC-phosphorylation; airway structure
INTRODUCTION

The mechanical stress imposed on the lungs during breathing is an important modulator of airway responsiveness in vivo(1; 6; 7). Our recent study demonstrated that continuous positive airway pressure (CPAP) applied to the lungs of non-anesthetized, tracheotomized rabbits for four days decreased lower respiratory system responsiveness to challenge with ACh(26). In addition, airway segments excised from the lungs of these animals and studied in vitro exhibited reduced contractility. However, the mechanism for this reduction in contractility was not determined. The stress-induced decrease in airway responsiveness could have resulted from alterations in the excitation-contraction coupling mechanisms of the smooth muscle cells, or it might reflect changes in the structure and/or composition of the airway wall tissues. Furthermore, although there are several studies evaluating the effects of chronic stress on isolated airway tissues(18; 21), there is no evidence that the observations made using in vitro models are relevant to the effects of imposing chronic stress on the airways in vivo, where they are exposed to systemic neural and humoral influences, as well as the chronic mechanical oscillations that occur during breathing.

In the present study, we assessed the effect of prolonged chronic stress of the lungs in vivo on airway smooth muscle force generation, MLC-phosphorylation and airway wall structure. To enhance the potential development of stress-induced structural changes, we applied mechanical stress for a prolonged period of time of 2 - 3 weeks. Our results suggest that chronic stress induces both structural and functional changes in the airways.
METHODS

Animal Preparation

Young ferrets (8 - 9 weeks, 0.47 - 0.6 kg.) were anesthetized (Isoflurane) and tracheotomized. As previously described, a custom 3.5 mm ID tracheostomy cannula (Bivona Medical Technologies, INC, CL27585) was sutured into the trachea and the flexible tubing from the tracheostomy connection was secured in place with a custom made vest worn by each animal(21; 26). Warmed and humidified air adjusted to a chosen level of continuous positive airway pressure was delivered to the airway (CPAP: Healthdyne Tranquility Plus; Fisher & Paykel Healthcare, MR 730). The connection at the top of the cage swiveled to provide tethering of the tubing and mobility for the animal within the cage. Tracheostomy care included instillation of 1 ml of sterile saline and suctioning every 6–10 hours to assist in removal of secretions that could block the cannula. Animals were randomly assigned to receive CPAP = 0 cmH2O (Low CPAP) or 6 cmH2O (High CPAP). The study protocol was approved by the Institutional Animal Care and Use Committee.

In-vivo measurements

High resolution computer tomography (HRCT): Using Isoflurane anesthesia and mild hyper-ventilation to induce a brief apnea that inhibited respiratory motion, HRCT images were obtained at an airway pressure of 0, 10, and 20 cmH2O. A GE LightSpeed Helical scanner system CT16 (GE Medical Systems, Milwaukee, WI, USA) with high resolution reconstruction algorithm, collimation thickness 0.625 mm, pitch 0.5 mm, 120 KV and 40 mA was used to
obtain the images, which were analyzed using software from GEAW (GE Medical Systems, Milwaukee, WI, USA) and EmphyxJ (Thoracic Imaging Group, Vancouver Hospital, Vancouver, Canada)(8; 21). There were 130-160 slices for each scan, which took approximately 12 seconds each. The field of view was 9.6 cm, and a pixel matrix of 512X512; pixel area was approximately 0.035 mm².

Respiratory System Responsiveness: Respiratory resistance was measured by forced oscillation during challenge with an inhaled aerosolized acetylcholine (ACh) dissolved in normal saline. Ferrets were anesthetized with thiopental sodium (50 mg/kg) and mechanically ventilated using a computer-controlled volume ventilator (Flexivent, SCIREQ Montreal, PQ) with a tidal volume of 8-10 ml/kg and a rate of 60 breaths per minute at a positive end-expiratory pressure of 4 cmH₂O. Before each measurement of resistance, the lungs were inflated four times to 30 cmH₂O to establish a standard volume history. Each inflation maneuver was held for several seconds and the total of 4 maneuvers took less than 20 seconds. Respiratory system resistance was calculated using a multiple-linear-regression of the pressure, flow, and volume signals recorded during a 1-Hz volume oscillation signal (15 ml/kg) that interrupted mechanical ventilation for one cycle. Resistance measurements were obtained every 3 seconds for 60 seconds following each Ach dose (1.0, 3.3, 10, 20, 33, 50 and 100 mg/ml). There was 8 minutes between starting each dose of Ach; the time included 1 minute of regular ventilation, TLC maneuvers, and adding next Ach dose to the nebulizer. The aerosol, which was produced with an ultra-sonic nebulizer (OMRON
Healthcare, INC. NE-U03V), was delivered into the inspiratory circuit and inhaled during 15 seconds of mechanical ventilation.

**In-Vitro Measurements**

**Isolated Lobe:** In a group of ferrets treated with High or Low CPAP, the left upper lobe was removed after euthanization of animals. The lobe was ventilated with Flexivent equipment using a rate = 60/min, PEEP = 5 cmH2O, and tidal volume proportional to the volume of the lobe relative to the total lung. Airway responsiveness to methacholine (MCh) was assessed using the protocol describe above with MCh concentrations of 1.0, 3.3, 10, 20, 33 and 50 mg/ml).

**Isolated Strips of Tracheal Smooth Muscle:** From the euthanized animals, a segment of the trachea was immediately removed and immersed in physiological saline solution (PSS) at 22°C (in mM: 110 NaCl, 3.4 KCl, 2.4 CaCl2, 0.8 MgSO4, 25.8 NaHCO3, 1.2 KH2PO4, and 5.6 glucose). PSS was aerated with 95% O2-5% CO2 to maintain pH 7.4. Smooth muscle strips (3 mm wide x 0.2–0.5 mm thick x 7 mm long) were dissected free of connective tissue and epithelium. Muscle strips were placed in PSS at 37°C in a 25-ml organ bath and attached to a force transducer(10; 11; 21). At the beginning of each experiment, the optimal length for muscle contraction was determined by progressively increasing the length of the muscle until the active isometric force elicited by ACh reached a maximum tension and the passive tension was approximately 0.5-0.6 gm. Cumulative concentration–response curves were constructed to step-wise increasing concentration of Ach (10\textsuperscript{-7.5} - 10\textsuperscript{-4}M).
Myosin light-chain (MLC) phosphorylation was measured as previously described(10; 11; 21). Muscle strips were rapidly frozen after contractile stimulation with 10^{-4} M ACh and then immersed in acetone containing 10% (wt/vol) trichloroacetic acid (TCA) and 10 mM DTT, which was pre-cooled using dry ice. Strips were thawed in acetone-TCA-DTT at room temperature and then washed four times with acetone-DTT. Proteins were extracted for 60 min in 8 M urea, 20 mM Tris base, 22 mM glycine, and 10 mM DTT. MLCs were separated by performing glycerol-urea-PAGE and transferred to nitrocellulose. The membranes were immunoblotted with polyclonal affinity-purified rabbit MLC-20 antibody. Unphosphorylated and phosphorylated bands of MLCs were detected using scanning densitometry. MLC phosphorylation was calculated as the ratio of phosphorylated MLCs to total MLCs.

**Airway Morphometry:**

After treatment with High or Low CPAP the animals were euthanized and the right lung was excised and fixed with 10% formalin at a distending pressure of 20 cmH_2O. From the formalin-fixed right lung, the main axial pathway was isolated by scraping the lung parenchyma away from the airways(13). The isolated airway was divided into airway segments, generation #4 - #9; the trachea was numbered generation #1 and the most distal isolated portion was generation #9. Each airway segment was embedded in paraffin and a 5-μm-thick section was cut from the distal portion of each segment. Tissues were stained with Masson's Trichrome and H&E. Tissues were visualized by light microscopy and images captured with a digital camera (SPOT, Diagnostic Instruments, Inc,
MI), and morphometric measurements from the fixed tissues were obtained from the digital images using imaging software (Metamorph, version 5.01r, Universal Imaging). The internal perimeter of the airway was calculated from the luminal border of the airway epithelium. The percentages of the airway wall occupied by epithelium, sub-epithelium, and smooth muscle were determined by point counting using a 30 x 30 grid superimposed on the digital image of the airway at a magnification of X100. The wall thickness was calculated as the average of measurements obtained in the four quadrants of each airway.

**Statistical Analysis:**

Dose response curves for High and Low CPAP treatment were compared using repeated measures analysis of variance (ANOVA) model with fixed effects for CPAP (H vs. L), agonist, and the CPAP by agonist interaction. Since there are multiple treatments, we compared differences at each dose in post-hoc analysis using p-values with Sidak adjustment to control for overall Type 1 error at the 5% level. The morphometric measurements versus airway generation for High and Low CPAP treatment were also compared by repeated ANOVA. Airway measurements from HRCT images for High and Low CPAP treated animals were compared with un-paired t-test, while the changes with CPAP treatment were compared by paired t-test.
RESULTS

Effects of chronic CPAP on airway reactivity

Ferrets were randomly assigned to receive chronic CPAP = 0 cmH₂O (Low CPAP; N = 5) or 6 cmH₂O (High CPAP; N = 5) for a period of 16-18 days. Following chronic CPAP treatment, pulmonary resistance was measured with the Flexivent before and during administration of progressively increasing doses of ACh (20, 33, 50, and 100 mg/ml). Baseline resistances did not differ for animals treated with High and Low CPAP (mean ± SE: 0.042 ± 0.006 vs. 0.060 ± 0.011 cmH₂O-s/ml; p = 0.20). Analysis of the dose response curve using repeated measures ANOVA demonstrated that inhaled ACh resulted in a progressive increase in resistance for both treatment groups; however the High CPAP group exhibited lower in vivo reactivity with smaller increases in resistances than the Low CPAP group (Figure 1). Analysis found significant differences between High and Low CPAP animals at ACh doses of 33, 50, and 100 mg/ml (p < 0.001).

In order to determine whether this decreased in vivo reactivity persisted ex-vivo, bronchial challenges were performed on left lower lobes excised from an additional group animals treated in vivo with High (N = 5) and Low CPAP (N = 4). The baseline airway resistances of the isolated left lower lobes did not significantly differ for the two groups (mean ± SE: 0.12 ± 0.054 vs. 0.07 ± 0.007 cmH₂O-s/ml; p = 0.36). Analysis of the dose response curve using repeated measures ANOVA demonstrated that inhaled ACh caused in an increase in resistance for both treatment groups; however, the High CPAP group exhibited lower in vivo reactivity with smaller increases in resistances than the Low CPAP
group (Figure 2). Analysis found significant differences between High and Low CPAP animals at ACh doses of 20, 33, and 50 mg/ml (p < 0.022, 0.002, 0.003).

In order to determine whether the decreased reactivity from in vivo mechanical stress was related to decreased contractility of the ASM, strips of tracheal smooth muscle were also isolated from animals treated in vivo with High or Low CPAP. Dose response curves showing force generation in response to ACh stimulation are illustrated in Figure 3A. Force increased with increasing concentrations of ACh for tracheal muscle strips obtained from both groups of animals. The force generation for the TSM strips removed from High CPAP treated animals was significantly lower than the force generated by TSM strips removed from Low CPAP treated animals; this difference was significant at ACh doses of 10^{-4.5} and 10^{-4} molar. In addition, the maximal dose of ACh resulted in a significantly lower level of myosin light chain phosphorylation in TSM strips obtained from animals treated with High CPAP compared to TSM strips from animals treated with Low CPAP (Figure 3B).

Effect of Chronic CPAP on Airway Size

The cross-sectional area of the distal tracheal lumen was measured from the HRCT images. Before CPAP, there was no significant difference in tracheal lumen area between the two groups (Figure 4). Following 2 – 3 weeks of High CPAP, there was a significant increase in the size of the tracheal lumen compared to before CPAP treatment, while there was not a significant increase in the size of the trachea lumen following Low CPAP treatment.
We also evaluated the length of the posterior tracheal membrane by measuring the distance between the ends of the cartilage ring at an airway pressure of 0 cmH₂O (Figure 5). Prior to CPAP treatment there was no significant difference in the lengths of the posterior membrane for the two groups of animals. Following treatment with High CPAP, the posterior tracheal membrane length increased significantly and it was significantly longer than the length of the tracheal membrane of the Low CPAP group. Low CPAP treatment did not significantly increase the length of the posterior membrane (Figure 6). There was no significant difference in the posterior tracheal length between the two groups at 20 cmH₂O.

The lumen area of airway generation #3, an intra-parenchymal airway, was also measured at an airway pressure of 0 cmH₂O. Following 2–3 weeks of treatment with CPAP, there was a significantly greater increase in lumen area of the High CPAP-treated group compared to the Low CPAP-treated group (Figure 6). Prior to CPAP treatment there was no significant difference lumen area between the two groups.

**Effect of Chronic CPAP on Airway Wall Composition**

Morphometric measurements of airway structure were made on formalin-fixed airways obtained from animals treated with High or Low CPAP. There were no significant differences between the two groups in airway lumen area, wall thickness, or the percentages of epithelium, sub-epithelium, and muscle within the airway wall (Figure 7).
DISCUSSION

Our study demonstrates that chronic mechanical distension of the lungs in vivo has a direct effect on the responsiveness of airway smooth muscle. We find that chronic mechanical distension of the lungs of conscious, active, tracheotomized ferrets for 2 - 3 weeks causes a decrease in pulmonary responsiveness to bronchial challenge with acetylcholine. Lobes isolated from animals after treatment with mechanical stress in vivo for 2 weeks also exhibit a reduction in pulmonary responsiveness to ACh stimulation, demonstrating that the reduction in responsiveness occurs in the absence of the vascular and neural influences present in vivo. Trachealis smooth muscle tissues isolated from animals subjected to high CPAP exhibit a depression of contractile responses to ACh stimulation and lower levels of MLC phosphorylation, indicating a direct effect of chronic stress in vivo on contractile protein activation and airway smooth muscle responsiveness that persists after the stress is removed. Thus the effects of stress on the activation of smooth muscle contractile proteins could underlie the decreased airway responsiveness observed in vivo. These observations suggest that mechanical forces experienced by the lungs in vivo for prolonged periods may result in significant persistent alterations in the airway smooth muscle responsiveness.

We also observed an increase in the luminal areas of the intra-thoracic trachea and intra-parenchymal airways, as assessed using HRCT in vivo, which indicates that prolonged administration of High CPAP results in larger airways. In addition, chronic exposure to High CPAP resulted in lengthening of the posterior
membrane of the trachea. The tracheal area assessed by HRCT was approximately 30% greater for High CPAP than Low CPAP animals. Assuming that baseline resistance is inversely proportional to the square of area, then the resistance of High CPAP animals would be estimated to be approximately 60% of the resistance of Low CPAP animals. Our measured baseline resistance values for High CPAP animals were approximately 67% of the values for Low CPAP animals. Although this difference was not statistically significant, it is consistent with our data obtained by HRCT.

We did not detect a significant increase in airway size in animals treated with High CPAP using the morphometric measurements of fixed airway sections. However, we expect that the sensitivity of using morphometric analysis to detect differences in airway size is less than that of the HRCT measurements. Unlike HRCT imaging, morphometric analysis of the airways cannot be obtained longitudinally on the same animal. In addition, more inter-subject measurement variability is involved in morphometric analysis due to factors such as tissue preparation and fixation, variations in the anatomical location of the isolated airways, and the angle of sectioning of the airway relative to its longitudinal axis, all of which can affect measured airway lumen size. We also observed no differences in the percentage of smooth muscle in the bronchial walls measured from cross-sectional images of the airways.

The effects on the airways we observed by chronically stressing the lungs of live breathing animals are analogous to previous observations made in isolated trachealis muscle strips and bronchial segments that were subjected to
chronic stress in vitro(18; 21). This is true despite the fact that the airways in vivo were subjected to chronic oscillations in stress caused by breathing and to the neural and humoral influences; whereas the muscles in vitro were subjected to static levels of elevated stress and no humoral influences. In addition, in isolated strips of TSM in vitro, either cyclic stress that mimics breathing or static stress can alter ASM contractility(22; 23). These findings suggest that the fundamental mechanisms that regulate the responses to chronic stress on the airways are local rather than systemic, and that in vitro models are useful for evaluating the mechanisms for the effects of chronic stress on airway smooth muscle function.

Effects of mechanical stress on the contractility of isolated tracheal muscle tissues in vitro have been attributed to reorganization of cytoskeletal and contractile proteins(2-4; 10; 14; 15). These processes may be initiated by mechano-sensitive protein complexes that localize to smooth muscle cell cytoskeletal/extracellular matrix adhesion junctions (2; 4; 9; 15). Mechanosensitive signals from these proteins within adhesion junctions could lead to the changes in myosin light phosphorylation and force generation that we observed in airways subjected to chronic stress in vivo. Cytoskeletal/extracellular matrix adhesion complex proteins, such as focal adhesion kinase and src, have been shown to regulate intracellular Ca\textsuperscript{2+} in both airway and vascular smooth muscle tissues (9; 16). Thus, mechanically induced alterations in the expression or activation level of these adhesion site proteins could result in changes in intracellular Ca\textsuperscript{2+}, and consequently alter myosin light chain phosphorylation, which regulates myosin activation and contractility. In
previous studies, the phosphorylation of focal adhesion kinase and its substrate, paxillin, were shown to be sensitive to changes in the mechanical load imposed on airway smooth muscle tissues (15; 25; 25).

The mechanisms for plasticity of the airway and ASM are likely to differ following acute and long term chronic stress of the tissue. The acute effects of strain may result from acute reorganization of the cytoskeleton system and contractile filaments, which has a relatively short time constant, while chronic strain may result in structural alterations resulting from changes in the composition or organization of tissue components, which would have a longer time constant. Although we did not observe morphometric changes in the airway wall at the light microscopic level, there may well be structural changes in connective tissue organization or composition, or cellular structures and their interactions with other tissue components that contribute to the functional changes that we observed.

In summary, our results demonstrate a direct connection between the decreased airway responsiveness caused by chronic mechanical stress of the lungs in vivo and a persistent decrease in contractile protein activation in the airway smooth muscle isolated from those lungs. The chronic stress also caused an increase in airway size, but no evident changes in the tissue composition of the airway wall. Chronic mechanical stress is known to be an important determinant of lung growth and development(4; 5; 12), which is also associated with a decline in airway reactivity(17; 19; 20; 24). The application of chronic
mechanical stress to the lungs may provide a useful therapeutic intervention for subjects with airway hyperresponsiveness.
FIGURE LEGENDS

**Figure 1**: In vivo dose response curves: Resistance (% Baseline) vs. Baseline (Ba), Saline (Sa), and increasing concentrations of acetylcholine (ACh) for animals treated with High CPAP (solid line; N = 5) and Low CPAP (dashed line; N = 5). High CPAP treated animals had significantly smaller increases in resistance than Low CPAP treated animals; the differences were statistically significant at the three highest concentrations of ACh (33, 50, 100 mg/ml: p < 0.0001).

**Figure 2**: In vitro dose response curves for isolated lobes: Resistance (% Baseline) vs. Baseline (Ba), Saline (Sa), and increasing concentrations of acetylcholine (ACh) for animals treated with High CPAP (solid line; N = 5) and Low CPAP (dashed line; N = 4). High CPAP treated animals had significantly smaller increases in resistance than Low CPAP treated animals; the differences were statistically significant at the three highest concentrations of ACh (20, 33, 50 mg/ml: p < 0.022, 0.002, and 0.003).

**Figure 3A**: In vitro dose response curves for isolated strips of Tracheal Smooth Muscle: Force (gm) vs. increasing concentrations of acetylcholine (ACh) for animals treated with High CPAP (solid line; N = 11) and Low CPAP (dashed line; N = 9). TSM from High CPAP treated animals had significantly lower force generation than TSM from Low CPAP treated animals; the differences were statistically significant at the two highest concentrations of ACh (p < 0.05).

**Figure 3B**: Myosin Light Chain (MLC) phosphorylation by TSM stimulated with $10^{-4}$ M ACh. There was significantly less MLC phosphorylation in TSM isolated from animals treated with High CPAP (N = 11) than TSM isolated from Low CPAP (N = 9) animals (p < 0.05).

**Figure 4**: In vivo measurement of tracheal lumen from HRCT images obtained at airway distending pressures of 0, 10, and 20 cmH$_2$O. Images were obtained pre and post CPAP treatment. Prior to treatment there were no significant differences in tracheal size for the High (N = 7) and Low (N = 4) CPAP groups. CPAP treatment resulted in a significant increase in the tracheal size for the High CPAP group (p < 0.05), but not for the Low CPAP group. Following CPAP treatment, High CPAP group had larger tracheal size than Low CPAP group (p < 0.05).

**Figure 5**: In vivo measurement of the length of posterior tracheal membrane from HRCT images at an airway pressure of 0 cmH$_2$O. Images were obtained pre and post CPAP treatment. Prior to treatment there was no significant differences in the length of the posterior tracheal membrane for the High (N = 7) and Low (N = 5) CPAP groups. CPAP treatment resulted in a significant increase in the length of the posterior tracheal membrane for the High CPAP group (p < 0.05), but not for the Low CPAP group. Following CPAP treatment, High CPAP group had a
longer length of the posterior tracheal membrane than Low CPAP group (p < 0.05).

**Figure 6**: In vivo measurement of the lumen area of the intra-parenchymal airway (generation #3) from HRCT images at airway pressure of 0 cmH₂O. Images were obtained pre and post CPAP treatment. Prior to treatment there was no significant differences in the size of the airway from High (N = 7) and Low (N = 4) CPAP groups. CPAP treatment resulted in a significant increase in the size of the airway for the High CPAP group (p < 0.05), but not for the Low CPAP group.

**Figure 7**: Comparison of morphometric measurements of formalin fixed airway segments from ferrets treated with High (N = 9) and Low (N = 7) CPAP. There were no significant differences between the two groups.
Figure 2

Resistance - Isolated Lobe (% Baseline)

Low CPAP
High CPAP

Mch (mg/ml)

Ba Sa 1 3 10 20 33 50
Figure 3A

![Graph showing the force (g) response to ACh (M) with data points for Low CPAP (n=9) and High CPAP (n=11). The graph includes error bars indicating variability.]

Figure 3B

![Bar graph comparing myosin light chain phosphorylation between Low CPAP and High CPAP groups. The graph shows significantly lower phosphorylation in the High CPAP group compared to the Low CPAP group.]

*Significant difference at p < 0.05.
Figure 4

![Graph showing tracheal lumen area (mm²) vs. pressure (cm H₂O). The graph includes four conditions: Pre-H (n=7), Post-H (n=7), Pre-L (n=4), Post-L (n=4). The conditions are compared for high CPAP and low CPAP. The graph indicates a significant difference (*High CPAP: pre vs. post).]
Figure 5

Length of Tracheal Membrane (mm)

- ** Pre vs. Post Low CPAP
- * Pre vs. Post High CPAP

Low CPAP

High CPAP
Figure 6

![Bar chart showing Gen # 3 Lumen Area (mm²) for Low CPAP and High CPAP conditions. The chart includes bars for PRE and POST measurements, with Low CPAP showing a smaller area compared to High CPAP. There is a note of significance marked with an asterisk (*) on the High CPAP POST measurement.]
Figure 7

- HCPAP (N=9)
- LCPAP (N=7)


