Increased mechanical strain imposed on murine lungs during ventilation in vivo depresses airway responsiveness and activation of protein kinase Akt

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Submitted 7 December 2012; accepted in final form 11 March 2013

Xue Z, Zhang W, Desai LP, Gao H, Gunst SJ, Tepper RS. Increased mechanical strain imposed on murine lungs during ventilation in vivo depresses airway responsiveness and activation of protein kinase Akt. J Appl Physiol 114: 1506–1510, 2013. First published March 14, 2012; doi:10.1152/japplphysiol.01460.2012.—Continuous positive airway pressure (CPAP) administered to tracheostomized rabbits and ferrets for 4 days or 2 wk suppresses bronchial reactivity in vivo and suppresses airway reactivity in lobes and tracheal segments isolated from these animals. In vitro studies of canine tracheal smooth muscle tissues indicate that mechanical loading suppresses the activation of the growth regulatory kinase, Akt, and that Akt is a negative regulator of smooth muscle differentiation. The transduction of mechanical signals in the tracheal tissues in vitro is mediated by integrin-associated adhesion complexes. To determine whether airway responsiveness and Akt activation are modulated by mechanical loads applied for short time periods to the airways of living animals in vivo, mice were mechanically ventilated for 2 h with high (5 cm H2O) or low (0–1 cm H2O) positive end-expiratory pressure (PEEP) and then ventilated at low PEEP for 30 min. Ventilation of mice with PEEP in vivo for 2 h depressed airway responsiveness to methacholine measured in vivo subsequent to the PEEP treatment. Airway narrowing in vitro in intraparenchymal airways in isolated lung slices and contractile responses of isolated tracheal segments in vitro were suppressed for at least 6 h subsequent to the in vivo exposure to PEEP. Tracheal segments isolated from high PEEP-treated mice exhibited significantly lower levels of Akt activation than tracheae from low PEEP-treated mice. The results indicate that mechanical loads imposed in vivo result in physiological and biochemical changes in the airway tissues after a relatively short 2-h period of in vivo loading.

mechanotransduction; airway smooth muscle; positive end expiratory pressure

THE MECHANICAL STRAIN IMPOSED on the lungs in vivo is an important modulator of airway responsiveness in vivo (8, 13, 17, 22, 23). We previously demonstrated that continuous positive airway pressure (CPAP) administered for 4 days or 2 wk to tracheostomized rabbits and ferrets suppresses bronchial reactivity in vivo and in isolated lobes removed from these animals (28–30).1 The contractility of tracheal smooth muscle tissues isolated from CPAP-treated animals is also depressed. Similarly, we found that nocturnal CPAP of 10 cm H2O administered to adults with asthma for 1 wk suppresses airway reactivity to methacholine challenge (5). We hypothesized that the effect of CPAP on airway responsiveness results from the increased mechanical strain on the airways, as the imposition of mechanical strain has been shown to decrease the subsequent responsiveness of isolated airway smooth muscle (ASM) tissues in vitro (26). In previous studies we showed that mechanically loading isolated canine tracheal smooth muscle tissues in vitro for 6 h can modulate the activation of nuclear signaling pathways that regulate protein synthesis (7, 27).

The imposition of a mechanical load on ASM decreases activation of the growth regulatory serine/threonine-specific protein kinase Akt. Akt is a downstream effector of integrin linked kinase (ILK), which localizes to adhesion sites in ASM tissues where it binds to the cytoplasmic tails of β-integrin proteins and regulates nuclear signaling pathways that determine the differentiation and phenotypic status of ASM (27). We found that the transcription of smooth muscle phenotype-specific proteins is negatively regulated by Akt; in contrast Akt stimulates ASM signaling pathways activated by interleukin (IL)-13 (7, 27). Mechanical stimuli modulate Akt activity in isolated ASM tissues, and this mechanotransduction process is mediated by integrin adhesion complexes (7, 27). However, there is currently no evidence as whether this signaling pathway is also sensitive to mechanical loads imposed on the lungs of animals in vivo. To determine whether this pathway is relevant in vivo, we used positive end expiratory pressure (PEEP) to mechanically load the airways of live mice for several hours and then assessed the effect of mechanical loading on airway responsiveness in vivo and on Akt activation in airways isolated from these animals.

MATERIALS AND METHODS

Positive end expiratory pressure ventilation protocol. The protocols for these studies were approved by the Indiana University Institutional Animal Care and Use Committee. Mice (C57BL/6) were anesthetized with pentobarbital sodium (30–50 mg/kg ip), tracheotomized, and mechanically ventilated (Flexivent, Scireq, Canada) at a rate of 300 beats/min and a tidal volume of 7–10 ml/kg. Mice were ventilated with either low positive end expiratory pressure (PEEP; 0–1 cm H2O) or high PEEP (5–6 cm H2O) for 2 h. As a single deep inspiration can have a transient bronchoprotective effect, we wanted to ensure that the more prolonged mechanical strain that we were imposing was producing a more persistent effect. Therefore, after treatment with high PEEP for 2 h, we reduced the high PEEP to the low PEEP level for 30 min. All animals were then ventilated for an additional 30 min at low (0–1 cm H2O) PEEP. Three total lung capacity (TLC) maneuvers with inflation to an inspiratory pressure of 20 cm H2O were performed every 15 min during the 2-h period of high or low PEEP.
In vivo respiratory responsiveness. At the completion of the PEEP protocol, airway responsiveness to inhaled methacholine (MCh) was assessed in vivo at 100% PEEP in both treatment groups. Respiratory resistance was measured by the computer-controlled ventilator by interrupting ventilation and imposing a 2.5-Hz sinusoidal signal and then resuming ventilation. Resistance was measured at baseline and following the nebulization of 20 μl of MCh for 10 s into the inspiratory side of the ventilator circuit. Increasing concentrations of MCh (10, 200, 300, and 400 mg/ml) were delivered and, following each MCh dose, resistance was measured every 30 s for the next 4 min to record a maximal response for each dose (16).

In vitro responsiveness of isolated tracheal segments. A second group of mice underwent the same in vivo protocol of mechanical ventilation with high or low PEEP. The mice were then euthanized, and the tracheas were removed and immediately immersed in physiological saline solution (PSS). Tracheal segments (5 mm) were dissected and attached to Grass force-displacement transducers in a tissue bath with PSS at 37°C within 30 min. Passive tension of the tracheal segment was set to maintain a preload of ~0.2 g. Isometric contractile force of the tracheal segments in response to acetylcholine (ACh) was measured for 2 min after stepwise increases in the concentration of ACh (10⁻⁷ to 10⁻³ M) (16).

We also evaluated the persistence of the effects of the administration of PEEP in vivo on tracheal segment contractility. Isolated tracheas from another group of mice that underwent the same PEEP protocol in vivo were maintained in the tissue bath for 3 h prior to measuring isometric force generation to ACh stimulation in vitro.

In vitro responsiveness of intraparenchymal airways. A group of mice underwent the in vivo protocol of mechanical ventilation with high or low PEEP. The mice were then euthanized, and the tracheas were removed and immediately immersed in physiological saline solution (PSS). The tracheas were then dissected and attached to Grass force-displacement transducers in a tissue bath with PSS at 37°C within 30 min. Passive tension of the tracheal segment was set to maintain a preload of ~0.2 g. Isometric contractile force of the tracheal segments in response to acetylcholine (ACh) was measured for 2 min after stepwise increases in the concentration of ACh (10⁻⁷ to 10⁻³ M) (16).

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In vitro responsiveness of intraparenchymal airways. A group of mice underwent the in vivo protocol of mechanical ventilation with high or low PEEP. The lungs were then inflated with 1 ml of 2% low melting point agarose at 37°C, rapidly chilled by infusing the pulmonary vasculature with ice-cold Hank’s balanced salt solution (HBSS), and removed from the chest and placed in ice-cold HBSS for 15 min to ensure gelling of the agarose. The lobes were separated and maintained chilled while sectioned into 130-μm-thick slices (Vibratome, Series 1000) (3, 9). The lung slices were then maintained in DMEM culture media at 37°C and 10% CO₂ for 4–5 h, which rewarmed the tissue and melted the agarose. Each lung slice was visualized with an inverted microscope (NIKON DIAPHOT, Nikon, Tokyo, Japan), and a single airway was selected for stimulation with 20 μl of 10⁻³ M ACh to assess airway narrowing. Images of the airway during stimulation were captured with a digital camera (SPOT RTKE Diagnostic Instrument). Airway narrowing was calculated by measuring the internal airway circumference and calculating the airway area pre-ACh stimulation and then repeating the measurement post-ACh stimulation. Measurements were performed using imaging software (Metamorph, version 5.01r, Universal Imaging). The contractile response to ACh was calculated as the percentage narrowing of the airway area from its prestimulation area.

Assessment of Akt activation. Another group of animals was subjected to the same PEEP protocol. The trachea were rapidly removed, rapidly frozen, and pulverized under liquid N₂. Pulverized trachea were mixed with extraction buffer containing 2% Triton X-100, 2 mM EDTA, pH 8.3, 20 mM Tris·HCl, pH 7.6, 0.4% SDS, phosphatase inhibitors (in mM: 2 sodium orthovanadate, 2 molybdate, and 2 sodium pyrophosphate), and protease inhibitors (in mM: 2 benzamidine, 0.5 aprotinin, and 1 phenylmethylsulfonyl fluoride). Each sample was centrifuged for the collection of supernatant and boiled in 4× Laemmli sample buffer. Proteins were separated using 7 or 8% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted for phospho 473 Akt and total Akt by incubating membrane with specific primary antibodies [polyclonal phospho ser473 Akt (Invitrogen) and polyclonal Akt (Cell Signaling)] at 4°C overnight followed by secondary antibodies for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence and quantified by scanning densitometry.

Statistical analysis. All dose-response curves were compared using repeated-measures ANOVA, which evaluated the effect of increasing agonist dose, the group effect of high vs. low PEEP treatment, and an interaction of agonist dose with treatment effect or a difference in the slopes of the dose-response curves for the two groups. Other analysis was done by paired or unpaired Student’s t-tests. A P < 0.05 was considered statistically significant.

RESULTS

In vivo respiratory responsiveness. Mice that had been ventilated under high (5 cmH₂O) or low (0 cmH₂O) PEEP were subjected to bronchial challenges with nebulized MCh (10–400 mg/ml), and resistance was measured following each dose. High PEEP-treated mice had a significantly smaller increase in resistance with increasing MCh dose compared with low PEEP-treated mice (P < 0.001) (Fig. 1). There was no significant difference between the baseline prechallenge resistances of high and low PEEP-treated animals. These findings demonstrate that exposure to high PEEP suppresses the response to subsequent MCh challenge.

Isometric force in response to ACh (10⁻⁷ to 10⁻³ M) was measured in tracheal segments removed from the mice that were exposed to high or low PEEP (Fig. 2). Tracheas were studied either 30 min (Fig. 2A) or 3 h (Fig. 2B) after removal from the mice. Contractile force in response to ACh was significantly suppressed in mice treated with high PEEP whether they were studied 30 min or 3 h after removal from the animal (Fig. 2). These findings demonstrate that the administration of high PEEP in vivo depresses the subsequent contractility of tracheal segments isolated from the treated animals and that this effect persists for at least 3 h.

Responsiveness of intraparenchymal airways in vitro. Lung tissue slices were prepared from the lungs of mice treated with low or high PEEP. Airway narrowing in response to ACh was studied in the tissue slices in vitro 5–6 h after removal from the animal; typically 21–36 airways were evaluated from each animal. The effect of PEEP treatment on percentage airway narrowing with ACh stimulation was assessed using analysis of variance.
covariance with baseline airway size as a covariate. Airways removed from high PEEP-treated animals exhibited significantly less airway narrowing with ACh stimulation than airways taken from low PEEP animals (Fig. 3). Preconstriction airway area was a significant covariate; smaller airways exhibited greater airway narrowing \((P < 0.001)\). However, there were no significant differences in the preconstriction airway lumen area for high and low PEEP-treated animals \((P = 0.63)\). These findings demonstrate that treatment with high PEEP has an effect on intraparenchymal airway narrowing that persists for at least 5–6 h after removal of the lungs from the animal.

### Effect of PEEP on Akt activation

Tracheal segments were isolated from high or low PEEP-treated mice, rapidly frozen, and evaluated for changes in the activation of Akt by immunoblot. Akt activation was assessed by probing Akt phosphorylation at serine 473 \((1)\). Tracheal tissue extracts from high PEEP-treated mice exhibited significantly lower Akt activation than tracheal extracts from low PEEP-treated mice \((P < 0.05;\) unpaired \(t\)-test) (Fig. 4).

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**DISCUSSION**

Our results demonstrate that the treatment of mice with PEEP in vivo for 2 h depresses airway responsiveness measured in the animals subsequent to the PEEP treatment. The effects of in vivo treatment with PEEP on the narrowing of isolated intraparenchymal airways and the contraction of isolated tracheal segments persist for at least 6 h after treatment. The persistence of the effect of PEEP on isolated airways suggests that mechanical load imposed in vivo directly alters ASM contractility. Our measurements of mechanosensitive Akt activation in airways isolated from mice are consistent with our previous observations of the effects of mechanical load imposed on canine ASM in vitro.

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**Fig. 2.** Isometric force in response to ACh \((10^{-7} \text{ to } 10^{-3} \text{ M})\) was measured in tracheal segments removed from the mice that were exposed to high or low PEEP \((\text{mean} \pm \text{SE})\). Tracheas were studied either 30 min \((A)\) or 3 h \((B)\) after removal from the mice. Tracheal segments assessed 30 min \((A)\) or 3 h \((B)\) after high PEEP treatment in vivo generated less force than tracheal segments assessed 30 min \((A)\) or 3 h \((B)\) after low PEEP treatment. \(n = 4\) for all 4 treatment groups \((P < 0.001\) for both \(A\) and \(B)\).

**Fig. 3.** ACh \((20 \mu \text{M} \text{ of } 10^{-3} \text{ M})\) induced narrowing of intraparenchymal airways \(N = 25–36\) airways/mouse) in lung slices from mice treated in vivo with high \((N = 3)\) or low PEEP \((N = 3)\) \((\text{mean} \pm \text{SE}\). A, images of an airway in lung slice removed from high and low PEEP-treated mice. ACh stimulation produced less airway narrowing in lung slice from high PEEP-treated animals compared with airway from low PEEP-treated animal. B, airway closure with ACh stimulation represents 100\% narrowing, whereas no constriction represents 0\% narrowing. Airways removed from high PEEP-treated animals exhibited significantly less airway narrowing in response to ACh stimulation in vitro than airways from low PEEP-treated animals \((P < 0.001)\). There were no significant differences in the preconstriction airway lumen area for high and low PEEP-treated animals \((P = 0.63)\).
ASM cell. The inhibition of Akt facilitates the nuclear signals that modulate the phenotype and function of the IPP-dependent mechanism (7, 27). Akt regulates nuclear mechanical load on ASM inhibits Akt activation via an in vitro and that mechanical loading inversely regulates the /H9251

responsiveness. 

ering of the airway wall and a persistent reduction in airway occur cumulatively over time, gradually leading to remod-

duction in airway contractility and a decrease in passive elasticity and myosin light chain phosphorylation in airways excised from the lungs of treated animals (29, 30). A 

Moreover, the chronic imposition of a mechanical load on the airways in vivo results in an increase in airway caliber accompanied by a decrease in both contractility and myosin light chain phosphorylation in airways subjected to chronic mechanical loading in vitro (20). Thus mechanical loading of airway tissues initiates changes in airway structure that persist and that may be responsible for the effects of loading on airway contractility. The signaling events triggered by an acute increase in mechanical load may initiate a cascade of cellular events that lead to structural and phenotypic changes in the ASM when the load is imposed for a prolonged time period. These events may occur cumulatively over time, gradually leading to remodeling of the airway wall and a persistent reduction in airway responsiveness.

We previously demonstrated that the integrin-associated ILK, PINCH, α-parvin (IPP) signaling complex plays a role in the transduction of mechanical signals imposed in ASM in vitro and that mechanical loading inversely regulates the activity of PI3 kinase-dependent Akt: an increase in the mechanical load on ASM inhibits Akt activation via an IPP-dependent mechanism (7, 27). Akt regulates nuclear signals that modulate the phenotype and function of the ASM cell. The inhibition of Akt facilitates the nuclear localization of the transcription factor, srf, which regulates the expression of smooth muscle phenotype-specific proteins. Akt inhibition induced by mechanical load also suppresses the synthesis and secretion of inflammatory cytokines by ASM (7, 27). Thus the IPP complex mediates signaling pathways that regulate genes that affect the structure and function of ASM (7). In the current study, we observed that mechanical loads imposed in vivo also decrease the activation of Akt. This suggests that the mechanotransduction pathways activated by mechanical loading in vivo may regulate nuclear signaling by mechanisms that are similar to those that we previously documented in tissues subjected to mechanical loading in vitro. Although we only examined changes in the activation of Akt, other adhesion complex proteins may also contribute to the regulation of pathways that modulate the structure and function of ASM in response to mechanical loading in vivo.

Our studies have documented the effectiveness of mechanical loading on the suppression of ASM contractility and airway responsiveness in vivo and in vitro and demonstrated its potential therapeutic effectiveness in animal models of asthma and in humans with asthma (5, 28–30). However, our in vitro studies of ASM tissues suggest that mechanical strain may provide further beneficial effects by suppressing airway inflammation (6, 7). These studies have shown that the imposition of mechanical strain on ASM tissues in vitro suppresses signaling pathways activated by the inflammatory mediator IL-13 that promote airway hyperresponsiveness and inflammation. We recently demonstrated that the administration of nocturnal CPAP depresses airway hyperresponsiveness in adults with asthma (5). This effect might partially be accounted for by the inhibitory effect of mechanical strain on the inflammatory responses of the airway tissues.

In our previous studies of the effects of mechanical loading on airway responsiveness in vivo, we loaded the airways for periods of 4 days to 2 wk (28–30). In the current study we found that mechanical loading for only 2 h resulted in a similar marked reduction in airway responsiveness that persisted for hours after the load was removed. Consistent with this, we were able to detect physiological and biochemical changes in the airway tissues for hours after this relatively short period of in vivo loading. This suggests that the administration of PEEP in vivo for a relatively short time period might have a beneficial therapeutic effect that could persist for prolonged time periods and that a much shorter period of CPAP might be effective in attenuating airway responsiveness in patients with asthma.

GRANTS

These studies were supported by National Heart, Lung, and Blood Institute Grants HL48522, HL29289 and HL074099 and American Lung Association and National Institutes of Health T32 postdoctoral fellowships to W. Zhang.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


