Inhibition of p21 Activated Kinase (PAK) Reduces Airway Responsiveness In Vivo and In Vitro in Murine and Human Airways

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Abstract

The p21-activated protein kinases (Paks) have been implicated in the regulation of smooth muscle contractility, but the physiologic effects of Pak activation on airway reactivity in vivo are unknown. A mouse model with a genetic deletion of Pak1 (Pak1⁻/⁻) was used to determine the role of Pak in the response of the airways in vivo to challenge with inhaled or intravenous acetylcholine (ACh). Pulmonary resistance was measured in anesthetized mechanically ventilated Pak1⁻/⁻ and wild type mice. Pak1⁻/⁻ mice exhibited lower airway reactivity to ACh compared with wild type mice. Tracheal segments dissected from Pak1⁻/⁻ mice and studied in vitro also exhibited reduced responsiveness to ACh compared with tracheas from wild type mice. Our findings demonstrate that Pak is a critical component of the contractile activation process in airway smooth muscle, and suggest that Pak inhibition could provide a novel strategy for reducing airway hyperresponsiveness.

Introduction

Asthma is characterized by repeated episodes of reversible airway obstruction and airway hyperresponsiveness to non-specific stimuli. An effective approach for the reduction of airway hyperresponsiveness and obstruction has been to inhibit airway smooth muscle contraction using bronchodilators. While beta-adrenergic bronchodilators have been the primary therapy for decades; increasing concerns about the long term safety and efficacy of these agents have led to a need for novel approaches to reduce airway smooth muscle contractility.

The p21-activated protein kinases (Paks) have been implicated in the regulation of cell motility and contractility in many eukaryotic cell types. We therefore hypothesized that Pak might provide a novel target for the reduction of airway hyperresponsiveness [1–5]. Although Pak has been previously implicated in the regulation of smooth muscle contractility, the physiologic effects of Pak activation on airway reactivity in vivo are unknown [6–8]. Pak 1, 2 and 3 isoforms are expressed in airway smooth muscle and have been implicated in the regulation of cytoskeletal dynamics in multiple cell types [9,10]. Pak1 is implicated in the regulation of smooth muscle cell motility and contraction and has been described as the dominant isoform in smooth muscle tissues [1,8,11]. In the present study, we used a mouse model with a genetic deletion of Pak1 to determine whether Pak regulates airway reactivity under physiologic conditions, and whether it could provide a target for the suppression of airway responsiveness [12]. We also tested the effects of a synthetic small molecule Pak inhibitor, IPA3, on airway reactivity in vivo [13]. IPA-3 is an allosteric Pak inhibitor that specifically inhibits the auto-activation of Pak isoforms 1, 2 and 3 [13,14]. In addition, we evaluated the effects of Pak inhibition on the responsiveness of murine and human airway smooth muscle tissues in vitro.

Our findings demonstrate that the suppression of Pak activity reduces airway responsiveness, and suggest that Pak could be an important molecular target for the suppression of airway reactivity in vivo.
Results

Expression of Pak1 isoform is inhibited in airway tissues from Pak1−/− mice

We evaluated the expression of Pak1, Pak2 and Pak3 in WT and Pak1−/− murine tracheae and isolated murine airway smooth muscle by immunoblot. Pak1 was detected in extracts from isolated tracheal smooth muscle tissues and whole tracheae from WT mice, but not in Pak1−/− mice (Figure 1A). Pak2 and Pak3 isoforms were also expressed at similar levels in tracheal smooth muscle from both WT and Pak1−/− mice, indicating that the expression of Pak2 and Pak3 were not altered in the Pak1 knockout mice (Figure 1A).

Genetic Deletion of Pak1 suppresses Pulmonary Responsiveness in vivo

We evaluated the role of Pak1 in the regulation of changes in pulmonary resistance in response to ACh in vivo by comparing responses of WT and Pak1−/− mice. Dose-response curves depicting increases in pulmonary resistance above baseline for increasing concentrations of inhaled ACh are illustrated in Figure 2A. There were no significant differences in the baseline resistances for either group. However, aerosolized ACh elicited significantly smaller increases in pulmonary resistance in response of WT and Pak1−/− mice compared to WT mice (P<0.05).

We also evaluated the effect of the route of agonist delivery upon airway responsiveness by performing dose-response curves in response to intravenous challenge with ACh (Figure 2B). Again, there were no significant differences for baseline resistances in animals challenged with intravenous ACh, and ACh elicited significantly smaller increases in pulmonary resistance in Pak1−/− mice compared to WT mice (P<0.05). The fact that the similar results were obtained using intravenous versus aerosolized ACh indicates the effect of Pak deletion on airway reactivity cannot be attributed to alterations in the epithelial barrier function in Pak1−/− mice.

Pharmacologic inhibition of Pak suppresses Pulmonary Responsiveness In Vivo

We evaluated the effect of a small molecule synthetic Pak inhibitor (IPA3) on pulmonary reactivity to ACh in WT mice in vivo. IPA3 allosterically inhibits the catalytic activity of Pak1, 2 and 3 isoforms [11,12]. Pak inhibitor IPA3 (20 µl of 5 µM, 1%DMSO) or Vehicle (1% DMSO) was nebulized into the ventilator circuit 60 minutes prior to bronchial challenge with aerosolized ACh. The respiratory response to inhaled ACh of WT mice receiving IPA3 was significantly decreased compared to that of WT mice that received Vehicle alone (Figure 3A).

In separate series of experiments, tracheae were isolated from WT animals that had received nebulized IPA3 in vivo to confirm the inhibition of Pak activation by IPA3. Phosphorylation of Thr 423 in the Pak catalytic domain occurs with its activation and is critical for its full catalytic function toward exogenous substrates [15,16]. Following removal of the epithelium from isolated tracheae, immunoblots were performed on tracheal extracts to measure the phosphorylation of Pak on tyrosine 423. ACh elicited a smaller increase in Pak phosphorylation at Thr 423 in WT animals compared to PK mice receiving IPA3 was significantly decreased compared to that of WT mice that received Vehicle alone (Figure 3A).

Genetic Deletion of Pak1 does not affect lung parenchymal structure or function

The effect of Pak1 deletion on the elastic properties of the lungs was evaluated by comparing pressure-volume curves obtained in WT and Pak1−/− animals in vivo. Mechanical ventilation was interrupted and the pulmonary system was slowly cycled between the end-expiratory pressure of 2.5 cm H2O and an airway pressure of 50 cm H2O. There were no significant differences in lung volume at 30 cm H2O between WT and Pak1−/− mice. There were also no differences in the pressure volume curves of the WT and Pak1−/− animals (Figures 5A, B).

Alveolar size was compared in lungs from WT and Pak1−/− animals by measuring mean linear intercepts from histological sections taken from lungs that had been formalin fixed at 30 cm H2O. There were no significant differences in mean linear intercept for WT and Pak1−/− animals, indicating that the dose of aerosolized IPA3 was effective at inhibiting Pak activation in vivo (Figure 3B).

Genetic Deletion of Pak1 does not affect airway structure

The effects of Pak1 deletion on the structure of the airways were evaluated by morphometric assessments of histologic sections of lungs from WT and Pak1−/− mice. There were no significant differences in the area of the airway wall or the areas of smooth muscle and epithelium (Figure 6).

A. Tracheal Smooth Muscle

WT Pak1−/

Pak1

Pak2

Pak3

B. Whole Trachea

WT Pak1−/

Pak1

Pak2

Figure 1. Pak1, Pak2 and Pak3 isoforms were detected in WT murine tracheal smooth muscle by immunoblot. No Pak1 was detected in extracts of isolated tracheal smooth muscle (A) or whole tracheae (B) from Pak1−/− mice. Immunoblots of tracheal smooth muscle were obtained from tracheal smooth muscle extracts pooled from 3 mice of each type. Whole trachea extracts were each from a single mouse.

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Pharmacologic inhibition of Pak inhibits Responsiveness of Murine Tracheal Segments in vitro

The effect of IPA3 on the contractility of murine tracheal segments to ACh was evaluated by concurrently stimulating pairs of tracheas with cumulative concentrations of acetylcholine (ACh) ($10^{-9}$ to $10^{-3}$ M) or 5-hydroxytryptamine (5-HT) ($10^{-3}$ to $10^{-8}$) and then measuring the isometric contractile force at each concentration. The ACh or 5-HT was then washed out and one trachea from each pair was treated with 100 μM IPA3 to inhibit PAK. The other trachea was treated with vehicle (1% DMSO). Tracheal segments were incubated in IPA3 or vehicle for 2 hours, after which a dose responsive curve to ACh or 5-HT was repeated. The contractile responses to ACh and 5-HT were significantly lower in tracheas treated with IPA3 compared to tracheas treated with vehicle (Figure 7).

Pharmacologic inhibition of Pak inhibits Responsiveness of Human Bronchial Rings in vitro

The effect of IPA3 on the contractility of human bronchial rings to ACh was evaluated by concurrently stimulating pairs of bronchial rings with cumulative concentrations of acetylcholine (ACh) ($10^{-9}$ to $10^{-5}$ M) as described above. The contractile responses to ACh were significantly lower in bronchi treated with IPA3 compared to bronchi treated with vehicle (Figure 8). The inhibition of PAK activation by IPA3 was confirmed by western blot for PAK T423 phosphorylation (data not shown).

Discussion

Our results demonstrate that inhibiting the activity of p21-activated protein kinase is an effective approach for reducing airway responsiveness in vivo. Mice with a genetic deletion of Pak1 exhibited significantly lower airway reactivity to acetylcholine in vivo, and the aerosol administration of a synthetic small molecule PAK inhibitor also reduced airway responsiveness in mice in vivo. Furthermore, isolated tracheal segments from Pak1−/− mice exhibited reduced contractility to ACh in vitro, and that this decrease in airway reactivity observed in vivo in Pak1−/− mice reflected a decrease in force generation by the airway smooth muscle. As no differences in the structure of the airways and lung parenchyma were detected in Pak1−/− and WT animals, decreased airway responsiveness did not appear to result from other factors that can alter airway responsiveness. Taken in sum, our observations provide strong evidence that the reduction of Pak activity lowers airway responsiveness in vivo, and that this effect is caused by a reduction in the contractility of the airway smooth muscle. The fact that Pak inhibition was also effective in reducing airway smooth muscle responsiveness in human bronchial tissues, which suggests that this approach could be effective in humans in vivo also.

We could not detect any differences in the structure or physical properties of the lungs from Pak1−/− mice as compared to WT mice. The quantity of airway smooth muscle within the airway wall was the same in the WT and Pak1−/− mice and airway morphometry was similar. There were also no differences between WT and Pak1−/− mice in lung parenchymal structure or function. These results suggest the lower level of airway reactivity in Pak1−/− mice are unlikely to be due to differences in lung structure. Previous studies that have evaluated the functions of other organ systems in Pak1−/− mice have also not detected major developmental abnormalities or organ defects [12,17,18]. Taken in sum, our findings in the Pak1−/− mice provide strong evidence that their decreased airway responsiveness in vivo results from the absence of Pak1 in their airway smooth muscle tissue, and that this reflects a critical role for Pak1 in the contractile activation of airway smooth muscle.

Figure 2. In vivo airway reactivity of Pak1−/− mice was lower than that of WT mice to aerosolized (A) and intra-venous (B) acetylcholine. Resistance in response to increasing concentrations of aerosolized acetylcholine (ACh) for wild-type (WT; N = 8) and Pak1 mice (N = 8) (A); values are means ± SEM. There was no difference in resistance at baseline. When analyzed by repeated measures ANOVA, resistance increased with increasing ACh dose (p = 0.0001), Pak1−/− mice had a significantly smaller slope of the dose response curve (p < 0.03), and a significantly smaller increase in resistance compared to WT mice (p < 0.03). Post-hoc analysis demonstrated Pak 1−/− compared to WT mice had significantly smaller resistances at all ACh concentrations (FIGURE 2A). Resistance in response to increasing concentrations of intravenous acetylcholine (ACh) for wild-type (WT; N = 4) and Pak1−/− mice (N = 4) (B); values are means ± SEM. There was no difference in resistance at baseline. When analyzed by repeated measures ANOVA, resistance for Pak1−/− mice increased less with increasing ACh dose (p < 0.0001) compared to WT mice. Post-hoc analysis demonstrated Pak1−/− compared to WT mice had significantly lower resistances at ACh concentrations (FIGURE 2B).
We showed that the synthetic Pak inhibitor, IPA3, was effective at inhibiting ACh-induced Pak activation in airway tissues both in vivo and in vitro. We also showed that IPA3 inhibits the contractile response of airway tissues to 5-HT in vitro, indicating that the effects of IPA3 are not agonist-specific. IPA3 is an allosteric Pak inhibitor that binds outside the ATP-binding pocket and specifically inhibits the auto-activation of Pak isoforms 1, 2 and 3 [13,14]. Treatment of WT mice with IPA3 inhibited airway responsiveness in vivo and in vitro similarly to that observed in the Pak12/2 mice. These results provide further support for our conclusion that the reduced airway responsiveness in Pak1−/− mice results from the loss of Pak1 activation in airway smooth muscle in response to agonist stimulation, rather than from an unidentified developmental defect caused by the absence of Pak1. IPA3 also inhibited the contractility of isolated human bronchial tissues to ACh, confirming that this inhibitor is also effective in human airway smooth muscle tissue.

This is the first study to demonstrate a role for Pak1 in the regulation of airway responsiveness in vivo. Previous studies that have evaluated the role of Pak in the regulation of smooth muscle contractile proteins have yielded inconsistent findings. Some of these studies have shown an inhibitory effect of Pak on contractile protein activation, suggesting that it would inhibit smooth muscle
Inhibition of PAK Reduces Airway Responsiveness

**Figure 5.** Comparison of the Lung Parenchyma (N = 3–5 in each group) for WT (black) and Pak1\(^{-/-}\) (grey) mice. There were no significant differences for lung volumes at 30 cmH\(_2\)O (p > 0.37) (A); pressure volume curves normalized to volume at 30 cmH\(_2\)O (B); or Alveolar Mean Linear Intercepts (MLI) (p > 0.40) (C).

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**Figure 6.** Comparison of Lung Histology for WT and Pak1\(^{-/-}\) mice. Airway wall area (A), airway smooth muscle area (B), and airway epithelium area (C) were not significantly different between the WT (black squares) and Pak1\(^{-/-}\) (grey circles) mice when assessed by ANOVA adjusting for airway perimeter (p > 0.15). (N = 5 mice in each group).

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contractility [9,17], whereas other studies indicate that Pak may contribute to the activation of contractile proteins and active tension development [8,12,13,19]. Most of these studies were performed using Triton skinned muscle tissues and exogenous recombinant Pak [5–7,16] or by expressing recombinant Pak in cultured cells [20]. The observations obtained using these preparations may not reflect the function of endogenous Pak in intact smooth muscle tissues under physiologic conditions. The results of our current studies evaluating airway responsiveness in vivo and in vitro clearly demonstrate that Pak is a critical intermediate in the contractile activation of airway smooth muscle and that its inhibition reduces airway responsiveness.

In conclusion, our findings demonstrate that Pak is an effective molecular target for inhibiting airway smooth muscle contraction and suppressing airway reactivity in vivo. This suggests that Pak inhibition could provide a novel strategy for reducing airway hyperresponsiveness in diseases such as asthma.

Materials and Methods

Ethics Statement

The protocols for these studies were approved by the Indiana University Institutional Animal Care and Use Committee (IACUC) and Indiana University Institutional Review Board, which waived the need for written consent for the use of surgical waste material for our research study and the tissue was, and remained de-identified.

Animals

Pak1−/− mice on a C57BL/6 background were backcrossed 6 generations as previously described [10,15]. Pak1−/− mice and Wild type (WT) C57BL/6 mice were studied at 8 weeks of age.

Human Airway Tissues

Fresh non-identified human lung tissue from surgical waste was collected within 1–2 hours of removal from the lungs and preserved in physiologic saline solution. Tissues were studied in vitro immediately after they were obtained.

In Vivo Pulmonary Measurements

WT and Pak1−/− mice were anesthetized with pentobarbital (6 mg/100 gm intra-peritoneal). Following tracheotomy, an 18-gauge cannula was inserted and securely tied with 4.0 braided silk. The animals were then mechanically ventilated with a computer-controlled small-animal ventilator (FlexiVent, SCIREQ, Montreal, Quebec) using a tidal volume of 8 mL/kg at a rate of 180 breaths/min and PEEP of 3.5 cm H2O. Pulmonary resistance was measured by the computer controlled ventilator by interrupting ventilation and imposing a 2.5 Hz sinusoidal signal and then resuming ventilation.

Airway Challenge

Following measurement of baseline resistance, animals underwent airway challenge with normal saline and then increasing concentrations of acetylcholine (ACh) or methacholine (MCh) by either aerosol challenge or intravenous challenge. For aerosol challenge, 20 μL of each solution of ACh ranging from 0.3 to 50 mg/ml was nebulized in line with the ventilation circuit over 10 seconds. Resistance was then measured every 30 seconds for the next 4 minutes to record a maximal response for each ACh dose. For intravenous Challenge, increasing volumes (3–100 μL) of acetylcholine (10 mg/ml) were infused through a jugular vein catheter for 20 seconds to deliver Ach doses ranging from 0.03–
1.0 mg. Measurements of resistance were obtained every 10 seconds for a minute and then the next Ach dose was administered.

**Evaluation of PAK inhibitor in vivo (IPA3)**

The effects of the small molecule Pak inhibitor IPA3 [13] on in vivo pulmonary responsiveness was evaluated in WT animals. IPA3 (5 mM) was dissolved in 1% DMSO and 20 μl was nebulized into the ventilator circuit 60 minutes prior to performing the Ach dose response curves. Control animals received nebulized 1% DMSO prior to bronchial challenge.

**Pressure-volume curves of the lungs**

The pulmonary elastic properties were evaluated from quasi-static pressure-volume curves obtained in vivo. Mechanical ventilation of the anesthetized animals was interrupted and the pulmonary system was slowly cycled between the end-expiratory pressure of 2.5 cm H2O and a maximal airway pressure of 30 cmH2O.

**Measurement of Contractility of Isolated Murine Tracheal Segments and Human Bronchial Rings In Vitro**

Pak1+/− and WT mice were euthanized with pentobarbital (12 mg/100 g intra-peritoneal) and intact tracheal segments were removed and dissected free of the epithelium. The isolated tracheal segments were mounted on wires and attached to Grass force transducers in tissue baths for the measurement of isometric force in response to increasing doses of Ach.

In some experiments, tracheas were isolated from WT mice to evaluate the effect of Pak inhibition on the contractile responses of airway tissues. Dose-response curves to Ach (10−9 to 10−3 M) or 5-hydroxytryptamine (5-HT) (10−9 to 10−5 M) were performed after 30 min incubation in physiologic saline. After washing the agonists from the tissues, one of each pair of the tracheal segments was treated with 100 μM IPA3 dissolved in 1% DMSO for 2 hours and the other segment was treated with 1% DMSO alone. A dose-response curve to Ach or 5-HT was then repeated on each pair of tissues concurrently.

Human bronchial rings (5 mm diameter, 2 mm wide) were dissected from lung specimens and cleaned of connective tissue. Rings were attached to force transducers as described above and dissected free of the epithelium. The isolated tracheal rings were mounted on wires and attached to Grass force transducers in tissue baths for the measurement of isometric force in response to increasing doses of Ach.

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Human bronchial rings (5 mm diameter, 2 mm wide) were dissected from lung specimens and cleaned of connective tissue. Rings were attached to force transducers as described above and equilibrated in a tissue bath for 30–60 min. Two rings were obtained from each lung specimen and studied concurrently. Dose-response curves to Ach (10−9 to 10−3 M) were performed after 30 min incubation in physiologic saline. After washing Ach from the tissue, one of the bronchial rings was treated with 100 μM IPA3 dissolved in 1% DMSO for 2 hours and the other ring was treated with 1% DMSO alone. A dose-response curve to Ach was then repeated on each tissue concurrently. Tissues from each group were quick frozen in liquid nitrogen for biochemical analysis to confirm the inhibition of Pak activation by IPA3.

**Analysis of protein expression and phosphorylation**

Proteins were extracted from pulverized murine tracheal smooth muscle, whole murine tracheal segments or human bronchial rings. Tissue extracts were then boiled in sample buffer (1.5% dithiothreitol, 2% SDS, 80 mM Tris-Cl (pH 6.8), 10% glycerol and 0.01% Bromophenol Blue) for 5 min, separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. Membranes were probed with antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (lg Jackson, ImmunoResearch Co.). Proteins were visualized by enhanced chemiluminescence (ECL). Antibodies against Pak1, Pak2, Pak3 and Pak Thr423 were obtained from Cell Signaling.

**Lung and Airway Histology**

Lung were excised and fixed with 10% formalin at a distending pressure of 20 cm H2O, sectioned into blocks, embedded in paraffin, and a 5-μm-thick section were stained with Masson’s Trichrome. 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). For airways, the internal perimeters were calculated from the luminal border of the airway epithelium. The cross-sectional areas of the airway wall, airway smooth muscle and epithelium were determined by point counting, as previously described [21]. For the parenchyma, alveolar size was quantified by measuring the mean linear intercept, which is the number of alveolar walls intersecting a line of known length [22,23].

**Author Contributions**

Conceived and designed the experiments: WCH JC DWC SJG RST. Performed the experiments: WCH WZ ZX HG. Analyzed the data: WCH. Contributed reagents/materials/analysis tools: JC DWC SJG RST. Performed the experiments: WCH WZ ZX HG. Analyzed the data: WCH. Contributed reagents/materials/analysis tools: JC DWC. Wrote the paper: WCH SJG RST.

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