Rapamycin decreases airway remodeling and hyperreactivity in a transgenic model of noninflammatory lung disease

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The mammalian target of rapamycin (mTOR) is a key regulator of cellular proliferation and survival. Treatment with the mTOR inhibitor rapamycin inhibits inflammation and AHR in allergic asthma models, but it is unclear if rapamycin can directly inhibit airway remodeling and AHR, or whether its therapeutic effects are entirely mediated through immunosuppression. To address this question, we utilized transforming growth factor-β1 transgenic mice null for the transcription factor early growth response-1 (Egr-1) (TGF-β1/TG/Egr-1+/- mice). These mice develop airway smooth muscle thickening and AHR in the absence of altered lung inflammation, as previously reported. In this study, TGF-β1/TG/Egr-1+/- mice lost body weight and developed severe AHR after 3 wk of lung-specific TGF-β1 induction. Rapamycin treatment prevented body weight loss, airway wall thickening, abnormal lung mechanics, and increases in airway resistance to methacholine after 3 wk of TGF-β1 induction. Increases in tissue damping and airway elastance were also attenuated in transgenic mice treated with rapamycin. TGF-β1/TG/Egr-1+/- mice on doxycycline for 8 wk developed severe airway remodeling. Immunostaining for α-smooth muscle actin and morphometric analysis showed that rapamycin treatment prevented airway smooth muscle thickening around small airways. Pentachrome staining, assessments of lung collagen and fibronectin mRNA levels, indicated that rapamycin also attenuated fibrotic pathways induced by TGF-β1 expression for 8 wk. Thus rapamycin reduced airway remodeling and AHR, demonstrating an important role for mTOR signaling in TGF-β1-induced/EGF receptor-mediated reactive airway disease.


Airway hyperreactivity (AHR) and remodeling are cardinal features of asthma and chronic obstructive pulmonary disease. New therapeutic targets are needed as some patients are refractory to current therapies and develop progressive airway remodeling and worsening AHR. The mammalian target of rapamycin (mTOR) is a key regulator of cellular proliferation and survival. Treatment with the mTOR inhibitor rapamycin inhibits inflammation and AHR in allergic asthma models, but it is unclear if rapamycin can directly inhibit airway remodeling and AHR, or whether its therapeutic effects are entirely mediated through immunosuppression. To address this question, we utilized transforming growth factor-β1 transgenic mice null for the transcription factor early growth response-1 (Egr-1) (TGF-β1/TG/Egr-1+/- mice). These mice develop airway smooth muscle thickening and AHR in the absence of altered lung inflammation, as previously reported. In this study, TGF-β1/TG/Egr-1+/- mice lost body weight and developed severe AHR after 3 wk of lung-specific TGF-β1 induction. Rapamycin treatment prevented body weight loss, airway wall thickening, abnormal lung mechanics, and increases in airway resistance to methacholine after 3 wk of TGF-β1 induction. Increases in tissue damping and airway elastance were also attenuated in transgenic mice treated with rapamycin. TGF-β1/TG/Egr-1+/- mice on doxycycline for 8 wk developed severe airway remodeling. Immunostaining for α-smooth muscle actin and morphometric analysis showed that rapamycin treatment prevented airway smooth muscle thickening around small airways. Pentachrome staining, assessments of lung collagen and fibronectin mRNA levels, indicated that rapamycin also attenuated fibrotic pathways induced by TGF-β1 expression for 8 wk. Thus rapamycin reduced airway remodeling and AHR, demonstrating an important role for mTOR signaling in TGF-β1-induced/EGF receptor-mediated reactive airway disease.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that functions as a key regulator of cell survival and proliferation. Activation of the mTOR pathway has been implicated in several human diseases characterized by dysregulated cell proliferation, including numerous cancers, tuberous sclerosis complex, and lymphangioleiomyomatosis (LAM) (15, 29, 34). Experimental studies demonstrated that rapamycin (also known as sirolimus), an inhibitor of mTOR, suppresses pulmonary fibrosis, as well as proliferation of pulmonary artery smooth muscle cells (31, 36). Rapamycin and its derivatives are used clinically as immunosuppressive agents to prevent transplant rejection (50). Rapamycin is similarly effective in inhibiting lung inflammation and AHR in the ovalbumin-induced model of allergic asthma, and preliminary studies support the utility of rapamycin in treatment of asthma (9, 13, 30). However, whether rapamycin can suppress airway remodeling and AHR independent of its immunosuppressive effects is unclear.

The pathogenesis of airway remodeling in asthmatic patients remains unclear, although a number of studies have suggested that epidermal growth factor (EGF) receptor (EGFR) signaling may play a role (1, 5, 7, 8, 25, 39). In a recent study by our group in adult mice, inhalation of the allergen dust mite increased EGFR activation, and both pharmacological and genetic inhibition of EGFR signaling attenuated allergen-induced increases in AHR and ASM (39). This demonstrated an important role for EGFR signaling, particularly in epithelial cells, in mediating the AHR and remodeling that develop as a result of chronic allergen treatment (39).

Activation of EGFR signaling in the lung by overexpressing the EGFR ligand transforming growth factor-α (TGF-α) in transgenic (Tg) mice primarily causes pulmonary fibrosis in the absence of any changes in inflammation (17–22). Recently, our laboratory has shown that TGF-α Tg mice lacking the transcription factor early growth response-1 (Egr-1) (TGF-α Tg/Egr-1+/- mice) also develop severe ASM thickening and AHR, as well as pulmonary fibrosis, in the absence of inflammatory changes (33). The goal of this study was to use this model to allow us to determine the efficacy of rapamycin treatment on airway remodeling, without the complicating effects of inflamm-
RATIONALE

Egr-1

our group, which showed that a 4 mg/kg dose for 6 days/wk was vehicle alone. The rapamycin dose was based on a previous study by

days/wk as an intraperitoneal injection at 4 mg/kg dissolved in vehicle/H9262 and CCSP-rtTA

Next, responses to methacholine were assessed using escalating doses

paraffin embedded, and cut into 5-

were anesthetized with a ketamine-xylaxine-acepromazine (4:1:1)

MATERIALS AND METHODS

Animal study protocols. Animal use protocols were approved by the Animal Care and Use Committee at the Cincinnati Children’s Hospital Research Foundation. CCSP-rtTA/(tetO)TGF-α/-/- Tg Egr-1/-/- mice (hereafter referred to as TGF-α Tg/Egr-1ko/ko mice) and CCSP-rtTA/(tetO)TGF-α/-/- Tg Egr-1/-/- mice (hereafter referred to as TGF-α Tg/Egr-1wt/wt mice) were generated as described previously (33). The CCSP-rtTA/(tetO)Tg mouse line used (line 1) drives expression of transgenes in Clara cells in the conducting airways, as well as a subset of type II cells (46). At 6 – 8 wk of age, Tg mice were placed on doxycycline (Dox)-containing chow (625 mg/kg) for 3 or 8 wk. CCSP-rtTA/(tetO)TGF-α/-/-/Egr-1/-/- mice not on Dox containing chow were used as controls (hereafter referred to as no-Dox controls). Previous studies have shown that AHR and responses in CCSP-rtTA

AHR and lung mechanics. A flexiVent mechanical ventilator system (SCIREQ, Montreal, Quebec, Canada) was used to assess baseline lung mechanics and changes in resistance due to methacholine inhalation, as previously described (3, 33). Briefly, a tracheotomy was performed with a 20-gauge blunt needle, the mice were connected to the flexiVent system, and baseline airway resistance data were collected after nebulization of 1× PBS, the vehicle for methacholine. Next, responses to methacholine were assessed using escalating doses (6.25, 12.5, 25, and 50 mg/ml) delivered by a nebulizer.

Immunohistochemistry and staining. Lungs were inflation fixed, paraffin embedded, and cut into 5-μm sections, and histochemistry and immunostaining were performed as previously described (32, 40). Antibody to α-smooth muscle actin (α-SMA; 1:10,000 dilution, clone 1A4; Sigma, St Louis, MO) was incubated overnight at 4°C with the sections. Movat’s pentachrome stain was also performed to assess fibrosis and remodeling. A Zeiss Axioplan 2 microscope (Carl Zeiss Microimaging, Thornwood, NY) was used to capture digital images of the stained sections.

Lung collagen content analysis and quantitative RT-PCR analysis of fibronectin mRNA. The Sircol collagen assay was used to assess pulmonary fibrosis, as previously described (18), on frozen left lung tissue. Lung RNA for quantitative RT-PCR was isolated using the SV Total RNA Isolation System (Promega, Madison, WI). cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer’s instructions. cDNA samples were amplified with TaqMan and a primer/probe set specific for fibronectin 1 (assay ID: Mm01256744_m1) (Applied Biosystems, Carlsbad, CA). Quantification of gene expression was analyzed by a 7300 Real-Time PCR System (Applied Biosystems) in triplicate, and expression levels were normalized to β-actin mRNA levels.

ASM morphometry. Immunostaining for α-SMA was performed as described previously (41). MetaMorph Imaging Software (version 6.2; Universal Imaging, Downingtown, PA) was used to measure ASM area (mm²) and internal perimeter (mm), as described previously (27, 33). Oblique cut airways were excluded from the analysis. The square root of ASM area was corrected to the internal perimeter of the airway (27, 33). Airways were divided into small (<700-μm internal perimeter), medium (700–1,200 μm), and large airways (>1,200 μm).

Data and statistical analysis. The Prism 4 software package (GraphPad Software, San Diego, CA) was used to analyze data and create graphs. Unpaired t-tests, one-way ANOVAs with Tukey’s post hoc test, or two-way ANOVAs with the Bonferroni post hoc test were used for statistical comparisons. P < 0.05 was considered significant.

RESULTS

Rapamycin prevents weight loss in TGF-α Tg/Egr-1ko/ko mice and changes in airway wall remodeling. To inhibit the mTOR pathway, TGF-α Tg/Egr-1ko/ko mice on Dox diet for 3 wk were treated with rapamycin. As shown previously, TGF-α Tg/Egr-1ko/ko mice lost body weight after 3 wk on Dox compared with no-Dox control mice and TGF-α Tg/Egr-1wt/wt mice on Dox (33). However, rapamycin-treated mice maintained their body weight, similar to no-Dox control mice (Fig. 1). Examination of lung tissue sections stained with hematoxylin and eosin confirmed, as reported in a previous study (33), that airway wall thickening occurs in TGF-α Tg/Egr-1ko/ko mice on Dox and in the absence of increases in inflammatory cells (Fig. 2).

The airway walls of rapamycin-treated TGF-α Tg/Egr-1ko/ko
mice did not show obvious signs of extreme thickening and remodeling (Fig. 2).

**Rapamycin prevents abnormal lung mechanics and increases in AHR in TGF-α Tg/Egr-1 ko/ko mice.** Studies using a flexiVent showed that TGF-α Tg/Egr-1 ko/ko mice on Dox for 3 wk developed abnormal lung mechanics and severe AHR, as previously described (33) (Fig. 3). In particular, airway resistance was higher in TGF-α Tg/Egr-1 ko/ko mice on Dox after inhalation of increasing doses of methacholine and reached statistical significance at the 50 mg/ml dose (Fig. 3). Rapamycin treatment blocked this increase in AHR in TGF-α Tg/Egr-1 ko/ko mice, and airway resistance to increasing doses of methacholine was similar in rapamycin-treated mice compared with no-Dox controls (Fig. 3). Rapamycin also prevented increases in airway elastance and tissue damping that develop in TGF-α Tg/Egr-1 ko/ko mice on Dox (Fig. 3).

**Rapamycin attenuates airway remodeling in TGF-α Tg/Egr-1 ko/ko mice.** As reported previously, TGF-α/Egr-1 ko/ko mice on Dox diet for 8 wk developed pulmonary fibrosis, as seen by pentachrome staining (Fig. 5) (31, 33). Rapamycin treatment attenuated this increase in fibrosis. Sircol assays also demonstrated that lung collagen levels increased 2.5-fold in TGF-α/Egr-1 ko/ko mice on Dox (Fig. 5). Rapamycin treatment reduced lung collagen levels by 32% compared with vehicle-treated TGF-α/Egr-1 ko/ko mice on Dox diet (Fig. 5). Fibronectin mRNA was detected and levels quantified using quantitative RT-PCR analysis. This showed that fibronectin mRNA levels were increased in TGF-α/Egr-1 ko/ko mice on Dox and reduced with rapamycin treatment (Fig. 6).

**DISCUSSION**

The intracellular signaling pathways that mediate airway remodeling are not well understood, although the mTOR pathway may be an important mediator downstream of growth factors and cytokines. In vitro studies suggested a role for the phosphatidylinositol 3-kinase (PI3K)-mTOR pathway, as inhibit...
bition of PI3K or mTOR reduced proliferation of ASM cells in response to EGF and PDGF stimulation (13, 35). Previous studies in mice and rats treated with ovalbumin to induce allergic asthma showed that the rapamycin analog SAR943 attenuated inflammation, AHR, and mucus cell hyperplasia (11, 13). However, since rapamycin greatly reduced the allergic inflammation in these studies, it is unclear if rapamycin’s effects were due to direct inhibition of AHR and remodeling, or whether its effects were due to reductions in inflammation and inflammatory cytokines. ASM thickening was also not assessed in these studies. In this study, we used TGF-β Tg/Egr-1ko/ko mice, which develop AHR and ASM thickening independent of inflammatory changes (33). This model enabled us to determine whether rapamycin can directly affect airway remodeling and increases in AHR without the complication of its effects on inflammation. Rapamycin treatment in this model not only reduced fibrosis, but also prevented increases in AHR and extensive ASM thickening, especially in the distal airways. These data indicate that mTOR plays an important role downstream of TGF-β-induced EGFR signaling, as rapamycin treatment prevented the severe AHR, ASM thickening, and pulmonary fibrosis that develops in this model when Egr-1 is absent (Fig. 7).

Recent clinical trials demonstrated therapeutic uses for rapamycin and its derivatives in human diseases characterized by dysregulated smooth muscle growth. Rapamycin-eluting cardiac stents reduced postangioplasty restenosis (14, 51). The recently completed Multicenter International LAM Efficacy of Sirolimus (MILES) trial showed that rapamycin improved lung function in patients with LAM, which is characterized by invasion of the lung by metaplastic smooth muscle-like cells (42). Here, we demonstrated that rapamycin reduced ASM thickening and AHR. Hence, these data show that rapamycin can inhibit ASM remodeling and AHR, independent of its effects on inflammation. This is also a proof of principle that modulating ASM independent of inflammation is a valid therapeutic strategy. Treatments that target airway remodeling in asthma will likely be of clinical use, since beneficial effects were reported in severe asthmatic patients treated with thermodplasty, which ablates ASM (10, 44).

This study suggests that inhibition of mTOR may directly inhibit airway remodeling and AHR, as well as have beneficial immunosuppressive effects. Severe asthmatic patients often suffer from a progressive lung disease, believed to be secondary to remodeling, with worsening lung function over time (26, 43). Although allergic asthma is the most common form of the disease, a sizeable subgroup of asthmatic patients are nonatopic and can develop apparently nonallergic AHR and airway remodeling with minimal eosinophilic inflammation (4, 45). Noninflammatory asthmatic patients can be especially difficult to treat with conventional immunosuppressive therapies (2, 45, 52). Alternatives to current therapies are urgently needed accordingly, inhibition of mTOR was proposed as an alternative treatment for severe refractory asthmatic patients dependent on chronic oral steroids (9).

mTOR’s effects are mediated via two distinct complexes, mTOR complexes 1 and 2 (mTORC1 and mTORC2) (48). mTORC1 is activated downstream of the PI3K/Akt pathway and then causes activation of ribosomal protein S6 to promote protein synthesis and subsequent cell growth and metabolism (23). In contrast, mTORC2 acts to phosphorylate Akt and regulate the cytoskeleton and cell proliferation; however, its upstream regulators are not clearly defined (23, 38). Rapamycin acts by binding the cytosolic FK-binding protein-12, which then interacts with the FRB domain of mTOR to inhibit mTORC1. Rapamycin cannot directly interact with mTORC2, which led to mTORC2 being referred to as “rapamycin insensitive” (38). However, prolonged (24 h) in vitro treatment with
rapamycin can lead to reduction in the levels of mTORC2 in many cell types and, thus, decreased Akt pathway signaling (49). Inhibition of mTORC1 is the most likely target of rapamycin in the present study, as previously our laboratory has shown (31) that, in TGF-β/H9251 Tg mice, rapamycin inhibited increases in phosphorylated P70 S6 kinase, which is downstream of mTORC1, but did not reduce levels of phosphorylated Akt, which is downstream of mTORC2. However, it is still possible that mTORC2 might have been affected in our study, as it is difficult to assess this in in vivo studies. Another limitation of this study is that we did not perform dose-response studies with rapamycin, and, therefore, it is possible that lower doses of rapamycin might be as effective as the one used in this study.

Rare pulmonary side effects have been reported in patients treated with rapamycin, including interstitial lung disease as well as pulmonary toxicity (16, 47). The possibility of severe pulmonary side effects raises some concerns regarding the potential clinical use of mTOR inhibitors, although the MILES trial investigating rapamycin use in LAM patients demonstrated overall beneficial pulmonary effects (42). Furthermore, chronic rapamycin treatment extended the life span of genetically heterozygous mice, likely by reducing the incidence of cancer and attenuating mechanisms of aging (24). The poten-

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**Fig. 4.** Airway smooth muscle (ASM) thickening in TGF-α Tg/Egr-1ko/ko mice after 8 wk on Dox diet is attenuated by Rapa treatment. A: α-SMA staining shows TGF-α Tg/Egr-1ko/ko mice have increased ASM after 8 wk on Dox diet. Rapa treatment reduced ASM around airways. Arrows indicate ASM. Bar = 100 μm. B: TGF-α Tg/Egr-1ko/ko mice on Dox diet for 8 wk develop severe ASM thickening. Morphometric analysis of ASM area (corrected to airway internal perimeter) in TGF-α Tg/Egr-1ko/ko mice showed decreases in ASM thickening in small airways (<700-μm internal perimeter) with Rapa treatment. ASM thickening around medium airways (700- to 1,200-μm internal perimeter) and large airways (>1,200-μm internal perimeter; C and D) was not significantly altered by Rapa treatment (P > 0.05, NS). Values are means ± SE; N = 4–6 mice per experimental group. *P < 0.05 vs. vehicle-treated no-Dox control mice by one-way ANOVA. ^P < 0.05 vs. vehicle-treated TGF-α Tg/Egr-1ko/ko mice by one-way ANOVA.
tial therapeutic efficacy must be considered in the context of side effects from therapy. Further investigations into the role of the mTOR pathway in lung remodeling may provide new therapeutic targets for patients with refractory reactive airway disease who are currently difficult to treat.

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Fig. 5. Rapa treatment reduces pulmonary fibrosis in TGF-α Tg/Egr-1ko/ko mice on Dox diet for 8 wk. A: TGF-α Tg/Egr-1ko/ko mice develop ASM thickening and pulmonary fibrosis after 8 wk of Dox diet. Pentachrome staining revealed reductions in lung remodeling with Rapa treatment. Bar = 100 μm. B: Sircol collagen assays showed increased total lung collagen in TGF-α Tg/Egr-1ko/ko mice on Dox diet for 8 wk. Rapa reduced lung collagen content in TGF-α Tg/Egr-1ko/ko mice. Values are means ± SE; N = 5–8 mice per experimental group. *P < 0.05 vs. no-Dox control mice by one-way ANOVA. +P < 0.05 vs. vehicle-treated TGF-α Tg/Egr-1ko/ko mice on Dox by one-tailed t-test.

Fig. 6. Rapa treatment reduces fibronectin mRNA levels in TGF-α Tg/Egr-1ko/ko mice on Dox diet for 8 wk. Lung levels of fibronectin mRNA were measured using quantitative RT-PCR analysis and corrected to β-actin mRNA levels. Levels of fibronectin mRNA were increased in TGF-α Tg/Egr-1ko/ko mice on Dox compared with no-Dox controls. Fibronectin mRNA levels were lower in TGF-α Tg/Egr-1ko/ko mice on Dox treated with Rapa. Values are means ± SE; N = 3–6 mice per experimental group. *P < 0.05 vs. vehicle-treated no-Dox control mice by one-way ANOVA. +P < 0.05 vs. TGF-α Tg/Egr-1ko/ko mice on Dox by one-tailed t-test.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES


