

Spontaneous Airway Hyperresponsiveness in Estrogen Receptor- α -deficient Mice

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Rationale: Airway hyperresponsiveness is a critical feature of asthma. Substantial epidemiologic evidence supports a role for female sex hormones in modulating lung function and airway hyperresponsiveness in humans.

Objectives: To examine the role of estrogen receptors in modulating lung function and airway responsiveness using estrogen receptor-deficient mice.

Methods: Lung function was assessed by a combination of whole-body barometric plethysmography, invasive measurement of airway resistance, and isometric force measurements in isolated bronchial rings. M2 muscarinic receptor expression was assessed by Western blotting, and function was assessed by electrical field stimulation of tracheas in the presence/absence of gallamine. Allergic airway disease was examined after ovalbumin sensitization and exposure.

Measurements and Main Results: Estrogen receptor- α knockout mice exhibit a variety of lung function abnormalities and have enhanced airway responsiveness to inhaled methacholine and serotonin under basal conditions. This is associated with reduced M2 muscarinic receptor expression and function in the lungs. Absence of estrogen receptor- α also leads to increased airway responsiveness without increased inflammation after allergen sensitization and challenge. **Conclusions:** These data suggest that estrogen receptor- α is a critical regulator of airway hyperresponsiveness in mice.

Keywords: lung function; asthma; hyperreactivity; M2 muscarinic receptor; estrogen receptor

A compelling body of evidence supports a role for female sex hormones in modulating lung function, airway hyperresponsiveness, and asthma in humans. Asthma prevalence rates are higher in women than in men between the ages of puberty and meno-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The effect of estrogens in the lung is highly controversial and the results of published studies are contradictory.

What This Study Adds to the Field

This study suggests that estrogen receptor- α is involved in airway hyperresponsiveness.

pause (1, 2). Menstrual cycle variations in pulmonary function and airway hyperresponsiveness have been well documented (3, 4). Females also appear to exhibit more severe airway hyperresponsiveness and more severe asthma than males (5, 6). The effect of estrogens in asthma is highly controversial and the results of published studies are contradictory. Both beneficial and detrimental effects have been reported (7, 8). For example, long-term and/or high doses of postmenopausal estrogen therapy have been reported to increase subsequent risk of asthma (7). In contrast, another study reported that supplemental estrogens could be used as steroid-sparing agents in women with asthma (8).

Airway hyperresponsiveness is one of the main features of asthma and is also a major risk factor for accelerated lung function decline, and hence the development of chronic obstructive pulmonary disease (COPD). The dominant autonomic control of airway smooth muscle in the lungs is provided by the parasympathetic nervous system (9, 10). The parasympathetic nerves release acetylcholine (ACh), which stimulates muscarinic M3 receptors on the smooth muscle to cause contraction. Concurrently, acetylcholine also stimulates M2 muscarinic receptors located on the nerves to limit further ACh release (9). Loss of M2 receptor function increases ACh release and potentiates vagally mediated bronchoconstriction (11). There is substantial evidence that loss of M2 muscarinic receptor expression and/or function on parasympathetic nerves is responsible for the development of airway hyperresponsiveness (9). Indeed, M2 muscarinic receptors are dysfunctional in subjects with asthma (12, 13) and in animal models of allergic airway disease (14, 15).

Estrogens mediate both transcriptional and nongenomic effects via α or β estrogen receptors (ERs). Both nuclear receptors are expressed in the lung, with ER β being more abundant than ER α (16), but their functions in this organ are largely unknown. Mice lacking either ER α (ER α knockout [α ERKO]) or ER β

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(β ERKO) have been developed using gene-targeting strategies (17, 18). The objective of the present study was to examine the role of ERs in modulating lung function and airway hyperresponsiveness. Our results show that ER α is a critical regulator of airway hyperresponsiveness and that α ERKO mice have reduced M2 muscarinic receptor expression and function in the lung. Hence, ERs could represent a novel therapeutic target for asthma and other diseases associated with reactive airways. Parts of this work have been published in abstract form (19).

METHODS

Mice

All procedures were performed under an approved animal study protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice (α ERKO, β ERKO, and wild-type littermate controls on a pure C57BL/6 background; 12–16 wk of age) were obtained from Taconic Farms (Germantown, NY). Further details about animals and treatments are provided in the online supplement.

Whole-Body Barometric Plethysmography

Basal lung function was measured in unrestrained mice using whole-body barometric plethysmography (Buxco Electronics, Wilmington, NC). Greater detail about these measurements is provided in the online supplement.

Respiratory Mechanics

Invasive analysis of lung function was performed on anesthetized mice using the Flexivent system (Scireq, Montreal, QC, Canada). Further detail is provided in the online supplement.

ACh Release Assay

ACh release was determined by spectrofluorometry using the Amplex Red ACh/acetylcholinesterase assay kit following the manufacturer's instructions (Molecular Probes, Carlsbad, CA). This assay is described in more detail in the online supplement.

Immunoblotting

Lung homogenates were analyzed by Western blotting for M2 muscarinic receptor expression, which was then normalized to actin expression by analyzing the M2 muscarinic receptor/actin band density ratio in each sample. Details are provided in the online supplement.

Assessment of M2 Muscarinic Receptor Function by Electrical Field Stimulation

Airway responsiveness to electrical field stimulation in the presence or absence of the M2 muscarinic receptor antagonist gallamine was assessed *ex vivo* as previously described (20), with some modifications that are described in detail in the online supplement.

Isometric Force Measurements in Isolated Bronchial Rings

The tension response in isolated bronchial rings to incrementally increasing concentrations of carbachol (10^{-7} to 10^{-3} M) was examined. Details are provided in the online supplement.

Allergic Airway Disease Model

Allergic airway disease was induced as previously described (21). Invasive lung function measurements were performed as described previously. Bronchoalveolar lavage (BAL) was performed and various endpoints examined as described in detail in the online supplement.

Statistical Analysis

Values for all measurements are expressed as mean $\pm$ SEM. Analysis of variance was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by unpaired two-tailed Student's *t* test. Statistics were performed using GraphPad Prism statistical software (version 4; GraphPad Software, Inc., San Diego, CA) and Microsoft Excel 2002 software. Significance levels were set at a *p* value of 0.05.

RESULTS

Baseline Lung Function and Airway Hyperresponsiveness

Whole-body barometric plethysmography. Whole-body barometric plethysmography was used to noninvasively assess baseline lung function in α ERKO and β ERKO mice. Breathing frequency was significantly reduced in male and female α ERKO mice relative to wild-type control animals (Table 1). Male wild-type mice were found to have a significantly higher tidal volume than female wild-type mice; however, this pattern was reversed in α ERKO mice (Table 1). Similarly, minute ventilation, peak inspiratory flow, and peak expiratory flow were higher in male versus female wild-type but not in α ERKO mice (Table 1). In contrast, disruption of ER β had no influence on sex differences in tidal volume, minute ventilation, peak inspiratory flow, and peak expiratory flow (Table 1). However, breathing frequency was significantly lower and peak inspiratory flow was significantly higher in female β ERKO mice relative to female wild-type mice. Moreover, tidal volume was higher in both male and female β ERKO mice relative to their sex-matched wild-type controls (Table 1). Together, these data suggest that both ER α and ER β play a role in the regulation of breathing, with ER α having the more dominant role.

α ERKO females exhibited substantially enhanced bronchial responsiveness to inhaled methacholine compared with wild-type females (Figure 1A). Similarly, male α ERKO mice were hyperresponsive to methacholine compared with their male wild-type counterparts, although the differences were less pronounced than in females (Figure 1B). In contrast, there were no

TABLE 1. BASAL LUNG FUNCTION IN WILD-TYPE, α ERKO, AND β ERKO MICE

	Wild-type Female	Wild-type Male	α ERKO Female	α ERKO Male	β ERKO Female	β ERKO Male
No. of animals	44	59	20	20	22	38
Body weight, g	22.0 $\pm$ 0.2	27.2 $\pm$ 0.3*	23.9 $\pm$ 0.6	26.9 $\pm$ 0.4	23.1 $\pm$ 0.4	28.9 $\pm$ 0.2
Penh	0.56 $\pm$ 0.01	0.56 $\pm$ 0.02	0.50 $\pm$ 0.02	0.50 $\pm$ 0.02	0.56 $\pm$ 0.02	0.56 $\pm$ 0.01
Frequency, breaths/min	507 $\pm$ 11	501 $\pm$ 11	468 $\pm$ 6*	447 $\pm$ 13†	471 $\pm$ 12*	495 $\pm$ 11
Tidal volume, μ l/breath	252 $\pm$ 7	277 $\pm$ 6*	275 $\pm$ 9*	256 $\pm$ 8†	293 $\pm$ 13*	311 $\pm$ 10†
Minute ventilation, μ l/min	127 $\pm$ 5	138 $\pm$ 5	128 $\pm$ 4	113 $\pm$ 6†	137 $\pm$ 8	150 $\pm$ 6
Peak inspiratory flow, ml/s	8.6 $\pm$ 0.3	9.9 $\pm$ 0.3*	10.0 $\pm$ 0.3*	8.8 $\pm$ 0.4†	9.6 $\pm$ 0.4*	10.4 $\pm$ 0.4
Peak expiratory flow, ml/s	6.0 $\pm$ 0.3	6.6 $\pm$ 0.3	6.0 $\pm$ 0.2	5.3 $\pm$ 0.2†	6.5 $\pm$ 0.3	7.1 $\pm$ 0.3

Definition of abbreviations: α ERKO = estrogen receptor- α knockout; β ERKO = estrogen receptor- β knockout.

Respiratory parameters were measured by noninvasive whole-body barometric plethysmography in wild-type, α ERKO, and β ERKO mice. Data represent means $\pm$ SEM.

* *p* < 0.05 vs. wild-type females.

† *p* < 0.05 vs. wild-type males.

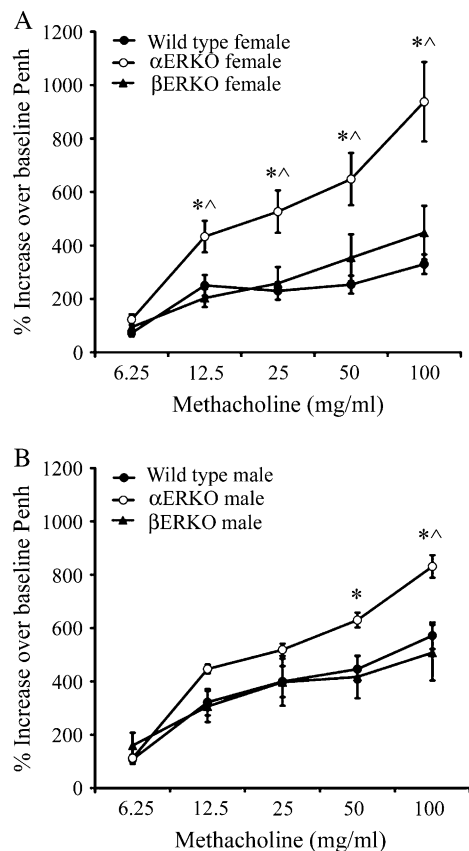


Figure 1. Airway responsiveness to inhaled methacholine in wild-type, estrogen receptor- α knockout (α ERKO) and β ERKO mice. Responsiveness to inhaled methacholine was measured using whole-body barometric plethysmography in awake, unrestrained (A) female and (B) male mice. Penh, an index of bronchoconstriction, was measured at baseline and after sequential delivery of increasing concentrations of methacholine (6.25–100 mg/ml). Results are reported as % increase in Penh over baseline values. Data represent means $\pm$ SEM of at least eight mice per group; * $p < 0.05$ versus wild type; ^ $p < 0.05$ versus β ERKO.

significant differences in methacholine responsiveness between male or female β ERKO mice and their sex-matched wild-type controls (Figures 1A and 1B). Because Penh (enhanced pause) is not a universally accepted measure of bronchoconstriction in mice (22), we also examined airway hyperresponsiveness using invasive techniques.

Invasive measurement of lung function and airway responsiveness. We focused our attention on the female α ERKO mice with the more robust lung phenotype. Lung function and methacholine responsiveness were measured in anesthetized, intubated, and mechanically ventilated mice. Under basal conditions, there were no significant differences between α ERKO and wild-type females with respect to total elastance (E), Newtonian resistance (Rn), or tissue elastance (H) (Table 2). However, total respiratory resistance (R), tissue resistance (G), and hysteresivity (η) were significantly reduced in α ERKO female mice compared with wild-type females (Table 2). The reductions in total respiratory resistance and in tissue resistance at baseline in the α ERKO females are consistent with the reduced baseline Penh as assessed by barometric plethysmography (Table 1). Consistent with the noninvasive barometric plethysmography results, invasive measurement of lung function confirmed that α ERKO females ex-

TABLE 2. BASAL LUNG FUNCTION IN WILD-TYPE AND α ERKO MICE MEASURED INVASIVELY

	Wild-type Female	α ERKO Female
No. of animals	9	10
R, cm H ₂ O-s/ml	0.63 $\pm$ 0.04	0.55 $\pm$ 0.03*
E, cm H ₂ O/ml	33.1 $\pm$ 0.7	33.4 $\pm$ 1.1
Rn, cm H ₂ O-s/ml	0.23 $\pm$ 0.02	0.25 $\pm$ 0.02
G, cm H ₂ O/ml	5.8 $\pm$ 0.2	5.1 $\pm$ 0.3*
H, cm H ₂ O-s/ml	31.5 $\pm$ 0.5	32.4 $\pm$ 0.9
η , G/H	0.186 $\pm$ 0.006	0.156 $\pm$ 0.005*

Definition of abbreviations: α ERKO = estrogen receptor- α knockout; η = hysteresivity; E = total elastance; G = tissue resistance; H = tissue elastance; R = total respiratory resistance; Rn = Newtonian resistance.

Respiratory parameters were assessed by invasive measurement of lung function in wild-type and α ERKO female mice. Data represent means $\pm$ SEM.

* $p < 0.05$ vs. wild type.

hibit hyperresponsiveness to inhaled methacholine (Figure 2). Specifically, PC₂₀₀(provocative concentration of methacholine aerosol which produces a 200% increase over baseline values)R, PC₅₀(provocative concentration of methacholine aerosol which produces a 50% increase over baseline values)E, and PC₂₀₀G were significantly reduced in α ERKO female mice relative to wild-type females, indicating that the lung periphery plays a role in the enhanced bronchoconstriction to inhaled methacholine in the α ERKO mice. α ERKO females also tended to have reduced PC₂₀₀Rn and reduced PC₅₀H, although these differences were not statistically significant. Together, these data confirm, via an alternative method, that lack of ER α leads to basal lung function abnormalities and hyperresponsiveness to inhaled methacholine.

Role of Circulating Estrogen in the α ERKO Phenotype

Female α ERKO mice have elevated circulating levels of estrogen and androgen (23). To address the possibility that altered sex hormone levels in the female α ERKO mice were responsible for

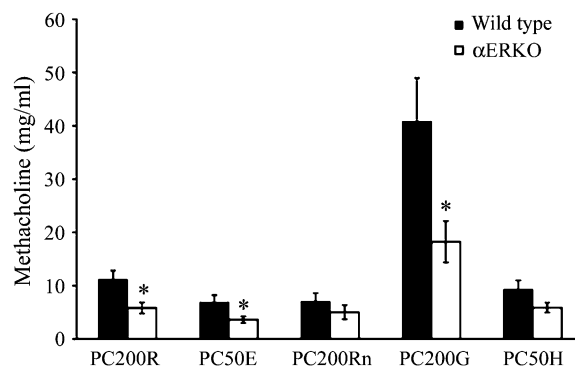


Figure 2. Invasive measurement of airway responsiveness in female wild-type and α ERKO mice. After acquisition of baseline data, airway responsiveness to inhaled methacholine (0–25 mg/ml) was assessed using the forced oscillation technique in anesthetized, intubated, and mechanically ventilated mice. Linear interpolation was used to determine the provocative concentration of methacholine aerosol at which a 200% increase (PC₂₀₀) over baseline values was observed for total respiratory resistance (R), Newtonian resistance (Rn), and tissue resistance (G), and at which a 50% increase (PC₅₀) over baseline values was observed for total elastance (E) and tissue elastance (H). Data represent the means $\pm$ SEM of at least six mice per group; * $p < 0.05$ versus wild type.

the methacholine hyperresponsiveness, wild-type and α ERKO female mice underwent ovariectomy and lung function was assessed 3 wk later using whole-body barometric plethysmography. Ovariectomy reduced absolute responsiveness to methacholine in the α ERKO mice (compare α ERKO mice in Figure 1A with α ERKO ovariectomized mice in Figure 3). However, removal of the ovaries failed to completely abolish differences in methacholine responsiveness between α ERKO and wild-type female mice because ovariectomized α ERKO mice were still hyperresponsive relative to ovariectomized wild-type mice (Figure 3). Interestingly, ovariectomy did not alter the response of wild-type mice to methacholine. Ovariectomy abolished most of the differences between wild-type and α ERKO mice with respect to basal lung function parameters (Table E1 of the online supplement). After ovariectomy, breathing frequency in α ERKO mice was still reduced relative wild-type mice, but this did not reach statistical significance ($p = 0.06$) (Table E1).

We next investigated the role of estrogen in modulating airway hyperresponsiveness using invasive measurements of lung function. Similar to the whole-body plethysmography experiments, we examined airway hyperresponsiveness in ovariectomized mice. In addition, to determine whether high levels of estradiol alone could recapitulate the hyperresponsive phenotype in wild-type mice, we administered estradiol using implantable, sustained-release pellets, which have been shown to produce circulating, steady-state estradiol levels comparable to those found in α ERKO female mice. In wild-type mice, neither ovariectomy nor estradiol treatment had any effect on airway responsiveness to methacholine (Figure 4A). In contrast, ovariectomy reduced responsiveness to methacholine in α ERKO mice as evidenced by increased values for PC₂₀₀R, PC₂₀₀G, and PC₅₀H (Figure 4B). Estradiol treatment had no potentiating effect on airway hyperresponsiveness in α ERKO mice. Collectively, these data suggest that the high circulating levels of estrogen in the α ERKO mice may contribute to the hyperresponsive phenotype; however, the data also suggest that high circulating estrogen levels alone are not sufficient for the phenotype to occur—the ER α must also be absent.

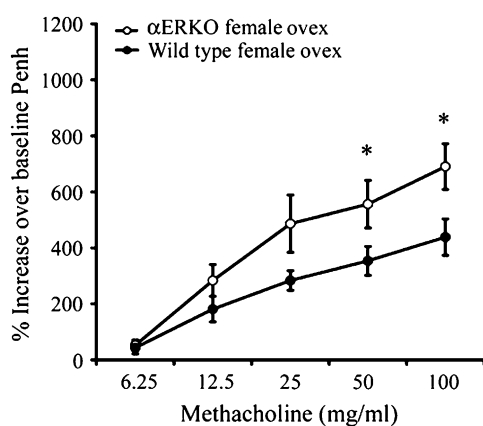


Figure 3. Airway responsiveness to inhaled methacholine in ovariectomized female wild-type and α ERKO mice. Responsiveness to inhaled methacholine was measured using whole-body barometric plethysmography in awake, unrestrained female mice. Penh, an index of bronchoconstriction, was measured at baseline and after sequential delivery of increasing concentrations of methacholine (6.25–100 mg/ml). Results are reported as % increase in Penh over baseline values. ovex = ovariectomized. Data represent means $\pm$ SEM of eight mice per group; * $p < 0.05$ versus wild-type ovex.

Role of Nerve and Muscle in Airway Hyperresponsiveness

ACh release after electrical field stimulation of tracheas. To assess the involvement of neural pathways in the hyperresponsive phenotype of α ERKO mice, we measured release of ACh from isolated tracheas subjected to electrical field stimulation. As shown in Figure 5, there was enhanced release of ACh from tracheas of α ERKO female mice relative to wild-type control animals. Because ACh release is controlled by presynaptic inhibitory M2 muscarinic autoreceptors (9), the increased release of ACh strongly suggests that these receptors are dysfunctional in the α ERKO mice.

M2 muscarinic autoreceptor function. We next investigated the function of the M2 muscarinic receptors. Tracheas were subjected to electrical field stimulation in the presence or absence of the specific M2 muscarinic receptor antagonist gallamine. Electrical field stimulation of tracheas from both genotypes resulted in frequency-dependent contractile responses. As predicted, pretreatment with gallamine potentiated the contractile response in tissues from wild-type mice (Figure 6A), demonstrating the presence of functional M2 muscarinic receptors. In contrast, pretreatment of α ERKO mouse tissues with gallamine had no effect on contractile response to electrical

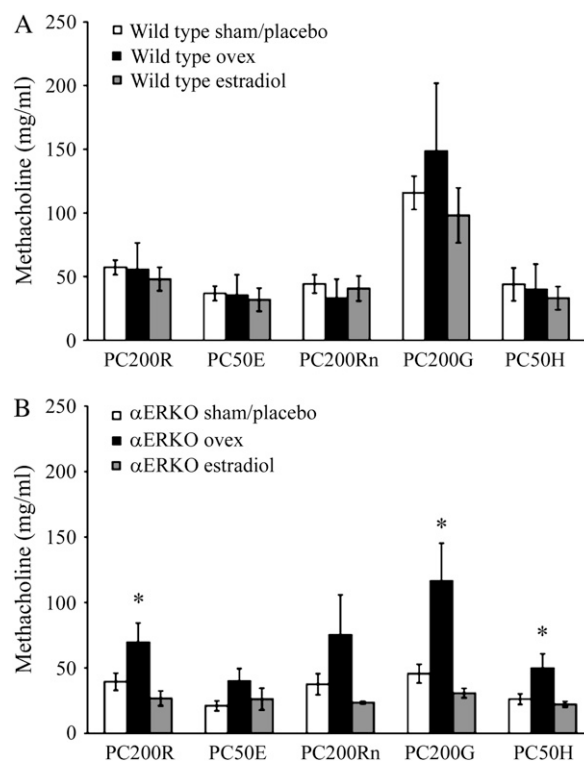


Figure 4. Invasive measurement of airway responsiveness to methacholine in female (A) wild-type and (B) α ERKO mice after ovariectomy (ovex) or implantation of sustained-release pellets containing estradiol. Control animals were sham ovariectomized or received placebo pellets and combined into one control group. After acquisition of baseline data, airway responsiveness to inhaled methacholine (0–25 mg/ml) was assessed using the forced oscillation technique in anesthetized, intubated, and mechanically ventilated mice. Linear interpolation was used to determine the provocative concentration of methacholine aerosol at which a 200% increase (PC₂₀₀) over baseline values was observed for R, Rn, and G, and at which a 50% increase (PC₅₀) over baseline values was observed for E and H. Data represent the means $\pm$ SEM of at least six mice per group; * $p < 0.05$ versus α ERKO sham/placebo.

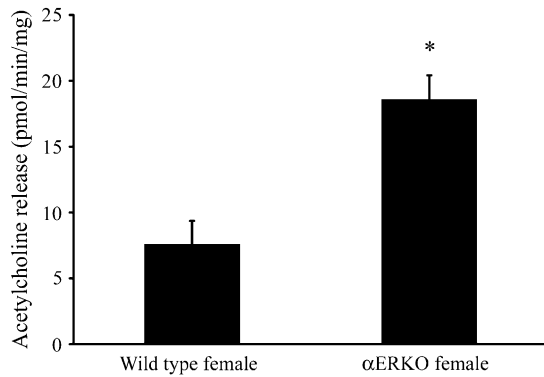


Figure 5. Acetylcholine release from wild-type and αERKO tracheas in response to electrical field stimulation. Data represent the means $\pm$ SEM of at least four mice per group; * p < 0.05 versus wild type.

field stimulation (Figure 6B). The lack of effect of gallamine on contractile response in αERKO mouse tissues indicates that αERKO mice have dysfunctional M2 muscarinic receptors. One possible mechanism for M2 muscarinic receptor dysfunction is down-regulation of M2 receptor expression (9). To examine this possibility, we measured protein levels of this receptor in lung homogenates using a specific M2 muscarinic receptor antibody. We found significantly reduced M2 muscarinic receptor expression in lungs from αERKO female mice relative to wild-type

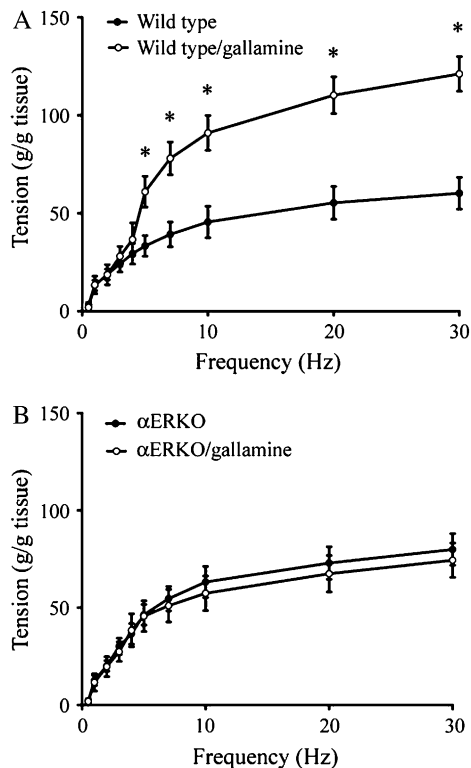


Figure 6. M2 muscarinic receptor function in wild-type and αERKO tracheas. The *ex vivo* response of tracheal smooth muscle from (A) wild-type female and (B) αERKO female mice to electrical field stimulation in the presence and absence of the M2 muscarinic receptor antagonist gallamine. Data represent the means $\pm$ SEM of at least eight mice per group; * p < 0.05 versus wild type.

control animals (Figure 7A). Densitometric analyses normalized to actin expression revealed an approximate 50% reduction in M2 muscarinic receptor expression in αERKO females (Figure 7B). Together, these data suggest that dysfunctional M2 muscarinic receptors may contribute to the hyperresponsive phenotype in αERKO mice.

Assessment of airway smooth muscle responsiveness. To assess the involvement of airway smooth muscle in the hyperresponsive phenotype of αERKO mice, isometric force measurements using isolated bronchial ring preparations were performed. Carbachol induced a significantly greater increase in isometric tension in female αERKO compared with female wild-type bronchial rings *ex vivo* (Figure 8). These data suggest that the hyperresponsive phenotype observed in αERKO female mice may be due, at least in part, to alterations in airway smooth muscle contractile function.

Airway responsiveness to serotonin. To determine whether the hyperresponsive phenotype of αERKO mice was specific to cholinergic agonists, we examined responsivity to serotonin. Similar to the results for methacholine, αERKO mice were hyperresponsive to inhaled serotonin, with significantly reduced PC₂₀₀R, PC₅₀E, PC₂₀₀Rn, PC₂₀₀G, and PC₅₀H relative to wild-type mice (Figure 9). These data suggest that αERKO mice are also hyperresponsive to other bronchoconstrictors.

Role of ERα in Allergic Airway Disease

Airway responsiveness after allergen challenge. Our next objective was to assess the role of ERα in a clinically relevant lung disease model. Airway responsiveness to methacholine was assessed in wild-type and αERKO mice in an established model of allergic airway disease involving initial sensitization and subsequent exposure to ovalbumin. Disruption of ERα had profound effects on the degree of airway hyperresponsiveness after allergen challenge (Figure 10). Thus, compared with allergic wild-type mice

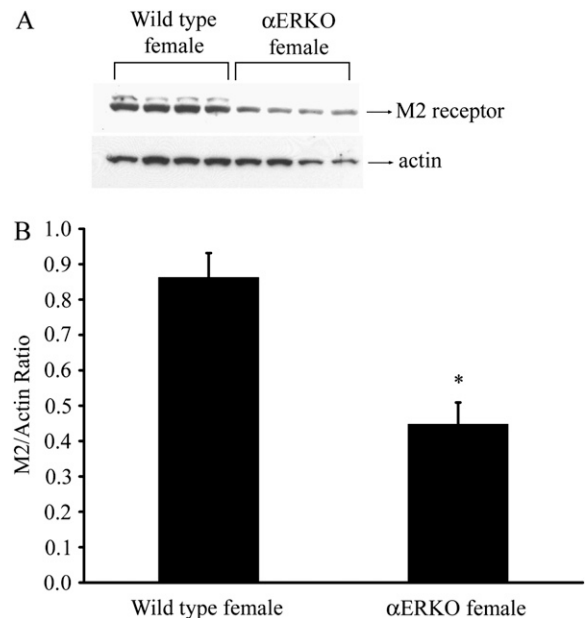


Figure 7. Pulmonary M2 muscarinic receptor expression in female wild-type and αERKO mice. (A) M2 muscarinic receptor and actin expression by immunoblotting in lungs from female wild-type and αERKO mice. (B) Density of M2 muscarinic receptor expression was normalized to actin expression in each sample. Data represent the means $\pm$ SEM of four mice per group; * p < 0.05 versus wild type.

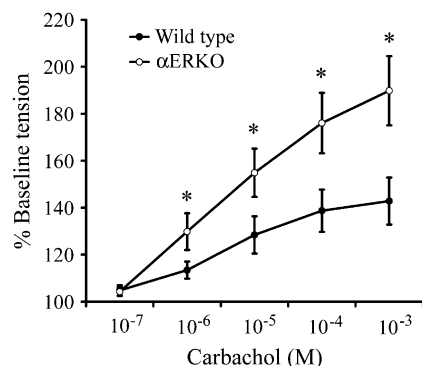


Figure 8. Isometric force measurements in isolated bronchial rings from female wild-type and α ERKO mice. The tension response of rings to incrementally increasing concentrations of carbachol (10^{-7} to 10^{-3} M) was examined. Data represent the means $\pm$ SEM of at least eight mice per group; * $p < 0.05$ versus wild type.

and nonallergic mice of both genotypes, allergic α ERKO mice exhibited significantly reduced PC₂₀₀R, PC₅₀E, PC₂₀₀Rn, PC₂₀₀G, and PC₅₀H. It should be noted that, in contrast to naive α ERKO mice, allergic α ERKO mice exhibited enhanced responsiveness to methacholine in the central airways as evidenced by significantly reduced PC₂₀₀Rn. These results indicate that absence of ER α leads to greatly enhanced airway responsiveness after allergen challenge.

Inflammation and cytokine release after allergen challenge. Allergen sensitization and exposure resulted in an influx of inflammatory cells into the airways. Interestingly, there were no differences between allergic wild-type and allergic α ERKO mice with respect to numbers of total cells, eosinophils, lymphocytes, and macrophages recovered in the BAL fluid (Figure 11A). There was a small reduction in the number of neutrophils in the airways of allergic α ERKO relative to allergic wild-type mice. There were no differences between allergic wild-type and allergic α ERKO mice with respect to tissue inflammation as assessed

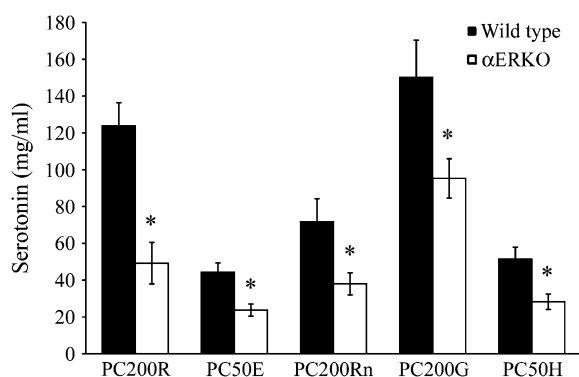


Figure 9. Invasive measurement of airway responsiveness to inhaled serotonin in female wild-type and α ERKO mice. After acquisition of baseline data, airway responsiveness to inhaled serotonin (0–20 mg/ml) was assessed using the forced oscillation technique in anesthetized, intubated, and mechanically ventilated mice. Linear interpolation was used to determine the provocative concentration of serotonin aerosol at which a 200% increase (PC₂₀₀) over baseline values was observed for R, Rn, and G, and at which a 50% increase (PC₅₀) over baseline values was observed for E and H. Data represent the means $\pm$ SEM of at least 10 mice per group; * $p < 0.05$ versus wild type.

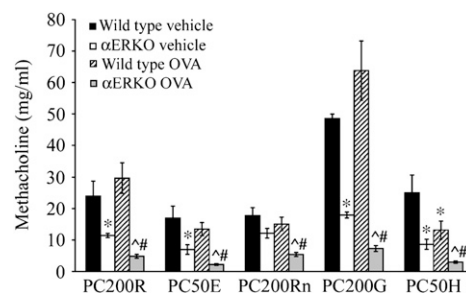


Figure 10. Airway responsiveness to inhaled methacholine after ovalbumin (OVA) sensitization and exposure in female wild-type and α ERKO mice. After acquisition of baseline data, airway responsiveness to inhaled methacholine (0–25 mg/ml) was assessed using the forced oscillation technique in anesthetized, intubated, and mechanically ventilated mice. Linear interpolation was used to determine the provocative concentration of methacholine aerosol at which a 200% increase (PC₂₀₀) over baseline values was observed for R, Rn, and G, and at which a 50% increase (PC₅₀) over baseline values was observed for E and H. Data represent the means $\pm$ SEM of at least 4 nonallergic and 12 allergic mice per group; * $p < 0.05$ versus wild-type vehicle; ^ $p < 0.05$ versus α ERKO vehicle; # $p < 0.05$ versus wild-type OVA.

histologically (Figure E1). There were no differences between allergic wild-type and allergic α ERKO mice in BAL fluid levels of IL-4, IL-5, IL-12, or tumor necrosis factor- α (Figure 11B). BAL fluid levels of total protein, a marker of alveolar epithelial permeability, were also similar in allergic wild-type and α ERKO mice (Figure 11C). These results suggest that lack of ER α does not appreciably alter the inflammatory response in the allergic airway despite having profound effects on the development of allergen-induced airway hyperresponsiveness.

DISCUSSION

The physiologic roles of ERs in the lung are largely unknown; hence, we examined lung function and airway hyperresponsiveness in ER-deficient mice. The results described herein implicate a major role for ER α in modulating lung function and airway hyperresponsiveness, and describe a potential mechanism by which ER α mediates airway responsiveness.

There is considerable evidence supporting a role for sex hormones in the neural control of breathing (24). Breathing disorders such as obstructive sleep apnea have been linked to sex hormone levels (24). There is an increase in sleep-disordered breathing after menopause, which can be alleviated by hormone replacement therapy (25, 26). Respiratory rhythm is generated by medullary neurons in the brainstem (27), a site in which ER α has been shown to be abundantly expressed (27–29). Interestingly, we found a marked reduction in breathing frequency in male and female α ERKO mice relative to wild-type control animals. Male wild-type mice have a significantly higher tidal volume than female wild-type mice; however, this pattern is reversed in α ERKO mice. Similarly, minute ventilation, peak inspiratory flow, and peak expiratory flow are higher in male versus female wild-type but not α ERKO mice. Together, these data indicate that functional disruption of ER α leads to changes in a variety of respiratory parameters and suggest that this nuclear receptor may be a critical regulator of breathing and respiratory rhythmogenesis in mice.

ER β disruption has no influence on sex differences in tidal volume, minute ventilation, peak inspiratory flow, and peak expiratory flow. However, breathing frequency is significantly lower and peak inspiratory flow is significantly higher in female

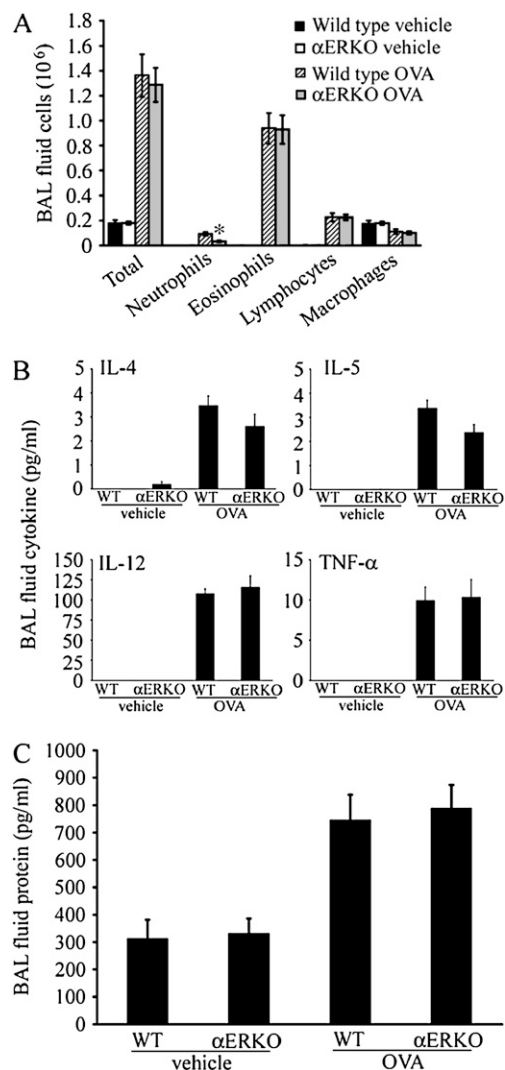


Figure 11. (A) Bronchoalveolar lavage (BAL) fluid cells, (B) cytokines, (C) and protein after OVA sensitization and exposure in female wild-type and α ERKO mice. BAL fluid was collected 24 h after the fifth daily OVA aerosol challenge. Data represent the means $\pm$ SEM of at least 3 nonallergic and 12 allergic mice per group; * $p < 0.05$ versus wild-type OVA.

β ERKO relative to female wild-type mice. Tidal volume is higher in both male and female β ERKO mice relative to their respective wild-type controls. Consistent with this observation, Massaro and Massaro recently reported that β ERKO mice have a higher body mass-specific lung volume relative to wild-type mice (30). These data suggest that ER β does play a role in the regulation of breathing, albeit a much less dominant role than ER α .

Airway hyperresponsiveness to cholinergic stimuli is a cardinal feature of asthma and a major risk factor for accelerated decline of lung function and development of COPD in humans (31, 32). The exact mechanism(s) underlying the development of airway hyperresponsiveness in chronic lung diseases such as asthma remains unknown. Several studies of risk factors associated with airway hyperresponsiveness have reported higher responsiveness in females compared with males (32–34), suggesting the involvement of sex hormones in the pathogenesis. Herein we demonstrate that in the absence of immunologic stimulation, α ERKO female mice exhibit substantially enhanced airway re-

sponsiveness to inhaled methacholine compared with wild-type females, suggesting that ER α is a critical regulator of this process.

Traditionally, airway hyperresponsiveness has been presumed to mainly involve the central airways and not the periphery. However, physiologic and pathologic evidence has emerged in recent years to support the role of the lung parenchyma and distal airways in the pathogenesis of airway hyperresponsiveness (35–38). Airway hyperresponsiveness is influenced by properties of the central airways and the surrounding pulmonary parenchyma, which is tethered to the airways, and by interactions between these two compartments (39). The exact location and precise mechanism for changes in tissue resistance are controversial, but many hypotheses have been proposed, including contraction of parenchymal interstitial cells, contraction of smooth muscle cells within alveolar ducts, and changes in the architecture of the alveoli and alveolar ducts (40–42). It has also been suggested that parenchymal changes could be secondary to airway narrowing, either by direct interaction between the airways and parenchyma or indirectly by altering lung volume (39). Invasive measurement of lung function in the α ERKO mice at baseline revealed hyperresponsiveness primarily in the periphery. After allergen challenge, there was marked hyperresponsiveness in both the central and peripheral airways. Interestingly, Massaro and Massaro recently reported that ERs are required for the formation of a full complement of alveoli in female mice (30). Thus, it is possible that structural abnormalities related to formation and size of alveoli may play a role in the abnormal hyperresponsive phenotype observed in these mice. Alternatively, the parenchymal defect in the α ERKO mice could be secondary to airway narrowing.

The reduced airway responsiveness to methacholine after ovariectomy of the α ERKO mice suggests that ovarian products may play a role in the hyperresponsive phenotype. However, the lack of an effect of estrogen supplementation or ovariectomy on airway responsiveness in wild-type mice suggests that estrogen alone is not the only culprit. One possible explanation for these findings may be that the absence of ER α allows ER β to predominate in this model. ER α and ER β have distinct expression patterns, with some organs having a predominance of one receptor over the other and other tissues having comparable expression of both receptors (16, 43, 44). Studies with α ERKO and β ERKO mice have revealed that these receptors have both overlapping and unique (and sometimes opposite) roles in mediating estrogen-dependent action *in vivo*. Both receptors are expressed in the lung, with ER β levels being higher than ER α levels (16). Studies have revealed that there is a complex interplay between ER α and ER β in the regulation and autoregulation of their respective promoters (44) and that some of the biological functions of one receptor may be dependent on the presence of the other receptor (16, 44, 45). In the present study, estrogen may produce different effects via ER β , and the observed hyperresponsive phenotype in α ERKO mice may be due to an altered estrogen response rather than an absent one, as has been postulated with other phenotypes displayed by these mice (46–48). An alternative possibility could be that the hyperresponsive phenotype is driven by an ovarian product other than estrogen, such as an androgen. In this regard, the ovaries of the α ERKO mice produce 17 β -hydroxysteroid dehydrogenase type III, an enzyme normally found only in testes, which converts androstenedione to testosterone. Indeed, α ERKO females have elevated plasma levels of androgens, similar to those seen in wild-type males (23).

Estrogen has been shown to modulate the density of muscarinic receptors *in vivo* in extrapulmonary tissues (49, 50), but there are no reports of estrogen modulation of muscarinic receptor expression or function in the lung. Importantly, expression

of the M2 muscarinic receptor is markedly reduced in α ERKO female mice relative to wild-type control mice. Consistent with this finding, tracheas from α ERKO female mice release more ACh in response to electrical field stimulation than tracheas from wild-type control mice. Furthermore, the lack of effect of gallamine, a selective M2 muscarinic receptor antagonist, on the contractile response of α ERKO tracheas to electrical field stimulation conclusively demonstrates M2 muscarinic receptor dysfunction in these mice. Together, these data indicate that one potential mechanism for airway hyperresponsiveness in α ERKO female mice could be down-regulation of M2 muscarinic receptor expression and function leading to increased ACh in the neuromuscular junction and resulting in enhanced bronchoconstriction after cholinergic agonist stimulation.

In light of our finding that α ERKO mice are also hyperresponsive to inhaled serotonin, it is possible that a reflex mechanism may be contributing to the generalized airway hyperresponsiveness in these mice. It is generally assumed that the response to methacholine reflects only a direct effect of the agonist on airway smooth muscle. However, studies suggest that a substantial component of the airway smooth muscle response to cholinergic agonists depends on a vagally mediated reflex (51). For example, administering cholinergic agonists increases the firing of sensory nerves in the lung, and vagotomy decreases the bronchoconstrictive response to methacholine (52, 53). Studies suggest that, in the mouse, the respiratory response to serotonin also involves a vagal reflex (54). Blockade of muscarinic M2 receptors with the subtype selective antagonist gallamine potentiates vagally induced bronchoconstriction by increasing ACh release (55). In addition, dysfunctional muscarinic M2 receptors have been shown to enhance reflex bronchoconstriction (56). Thus, if reflex bronchoconstriction is contributing to the enhanced airway hyperresponsiveness to methacholine and serotonin, then dysfunctional M2 receptors could be playing a major role by leading to enhanced ACh release after vagal stimulation. The *in vitro* response to carbachol may reflect an additional smooth muscle defect and this may or may not contribute to the airway hyperresponsiveness observed in the intact animal. The combined defects at the level of smooth muscle and nerve may explain why the hyperresponsiveness is so prominent in the α ERKO female mice.

Although deletion of ER α has minimal effects on airway inflammation after allergen challenge, it has a profound effect on the development of allergen-induced airway hyperresponsiveness. Indeed, the differences in airway responsiveness between wild-type and α ERKO mice are even more pronounced after exposure to ovalbumin. Although airway inflammation is frequently correlated with airway hyperresponsiveness to methacholine, and treatment of inflammation often improves hyperresponsiveness, dissociation between airway inflammation and hyperresponsiveness has been observed in other murine models of allergic airway disease (57, 58). Of note, allergen challenge induced minimal changes in airway responsiveness in wild-type mice in our study. This is not surprising because other investigators have shown that the C57BL/6 strain is one of the least responsive strains in terms of the development of airway responsiveness after ovalbumin sensitization and exposure (59, 60). Indeed, the fact that allergen challenge induced such a profound degree of airway hyperresponsiveness in α ERKO mice on a C57BL/6 background indicates that ER α is a potent regulator of this process.

Although targeted disruption of specific genes in mice offers a powerful tool to investigators, caution must be exercised in extrapolating from these studies to physiologic effects in humans. Knockout of a gene in mice may produce different effects than inactivation of the same gene in humans. In addition, targeted

disruption in mice may lead to compensatory changes in expression of other genes, which may hamper interpretation of the results. Nevertheless, it is of interest to speculate on the potential clinical significance of our findings. There are several polymorphic sites in the human ER α locus and some of these polymorphisms have been associated with diseases such as cancer and osteoporosis (61, 62). Interestingly, in some instances, the phenotype associated with the polymorphism is also dependent on high levels of estrogen (62). The role of hormone replacement therapy in the treatment and prevention of cardiovascular disease has been highly controversial due to conflicting findings. It has been suggested that variation in estrogen effects on the cardiovascular system may be related, at least in part, to common variants in the ER α gene, which can significantly alter the way a person responds to endogenous and exogenous estrogen (63). It is possible that the conflicting human data on estrogen effects in the lung could also be due to genetic variability in ER α . Indeed, genetic variability in ER α with resultant effects on estrogen sensitivity could underlie the greater incidence and severity of asthma and airway hyperresponsiveness in females between the ages of puberty and menopause when circulating estrogen levels are high, and could also underlie the phenomenon of premenstrual asthma as estrogen levels fluctuate during the menstrual cycle. Of interest, the relevance of our findings to humans is supported by a recent publication by Dijkstra and coworkers who found that variations in ER α were associated with airway hyperresponsiveness and more rapid lung function decline in subjects with asthma (64).

In conclusion, our data suggest that, in the mouse, lack of ER α leads to airway hyperresponsiveness via defects at the level of airway smooth muscle and nerves, and may involve regulation of M2 muscarinic receptor expression and function. Our data also suggest that the α ERKO mouse may prove to be a useful model to study the mechanisms of sex hormone modulation of airway responsiveness in humans.

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

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Spontaneous airway hyperresponsiveness in estrogen receptor- α deficient mice

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Online Data Supplement

Methods

Mice. All animals were maintained in plastic cages under a 12-h light, 12-h dark schedule in a temperature-controlled room (21-22°C) and fed NIH 31 mouse chow and fresh water *ad libitum*. In ovariectomy studies, ovaries were removed three weeks prior to experimentation to allow the levels of endogenous ovarian steroids to decrease. Control mice were sham ovariectomized. In studies involving estradiol treatment, pellets (0.05 mg estradiol/21 day release) purchased from Innovative Research of America were implanted subcutaneously in the shoulder region. Similarly, control mice were implanted with placebo pellets. Experiments involving pellet implanted mice were performed 2 weeks post-implantation. This particular pellet was chosen because it produces circulating levels of estradiol in wild type mice which mimic levels found in α ERKO mice (1-3).

Whole body barometric plethysmography. Basal lung function was measured in unrestrained mice using whole body barometric plethysmography (Buxco Electronics). From each chamber, a pressure signal was generated from the pressure difference between the main chamber containing the unrestrained mouse and a reference chamber. Signals were analyzed using BioSystem XA software (Buxco Electronics) to derive whole body flow parameters including respiratory frequency, tidal volume, minute ventilation, peak inspiratory flow, peak expiratory flow and Penh (enhanced pause). Penh is a unitless parameter that strongly correlates with lung resistance (4). Signals were recorded every 6 seconds for 60 minutes and the values were averaged. Airway responsiveness to inhaled methacholine aerosol was then assessed. After measurement of baseline Penh for 10 minutes, changes in Penh in response to increasing doses of methacholine (0, 6.25, 12.5, 25, 50 and 100 mg/ml) were measured. Penh was recorded every 6 seconds during the 3 minute aerosol delivery and for 5 minutes following each aerosol

delivery. Penh values were averaged over the 5 minute response time only and are expressed as % increase in Penh over Penh response to saline aerosol.

Respiratory Mechanics. Invasive measurements of lung function were performed using the FlexiVent system (Scireq) and the forced oscillation technique (5). This FlexiVent system is a platform that integrates a computer-controlled small animal ventilator with measurements of respiratory mechanics. Using this system, two models were employed to assess respiratory mechanics: 1) the linear first-order single compartment model, and 2) the constant phase model. The single compartment model which is the standard model of respiratory mechanics, was employed to determine total respiratory resistance (R) and elastance (E). The constant phase model is an advanced model of respiratory mechanics that offers parametric distinction between central and peripheral respiratory mechanics and which was introduced by Hantos et al. (6). This model was employed to determine Newtonian resistance (R_n , a measure of central airways resistance), tissue damping (G, a measure of resistance in the peripheral airways and parenchyma), and tissue elastance (H, a measure of elastance in the peripheral airways and parenchyma). This model has been employed by numerous investigators (7-10).

Mice were anesthetized with urethane (1.5 g/kg i.p.). Once surgical anesthesia had been established, a tracheostomy was performed and a 19 gauge stainless steel cannula was inserted into the trachea. Animals were then paralyzed with pancuronium bromide (0.8 mg/kg i.p.) and placed on a 37°C heating pad, and the cannula was connected to the computer-controlled flexivent ventilator. Ventilation was maintained at a rate of 150 breaths/minute and a tidal volume of 7.5 ml/kg, with a positive end-expiratory pressure of 3 cm H₂O. All data points were determined by the FlexiVent software (version 4.0) by using multiple linear regression to fit each data point to the single compartment or the constant phase model, as appropriate. Baseline values

for each mouse were obtained by applying a 2 s perturbation at a frequency of 2.5 Hz followed by an 8 s pseudo-random perturbation consisting of waveforms of mutually prime frequencies (0.5 to 19.6 Hz) a total of 3 times at 30 s intervals; these maneuvers generated data that were fit to the single compartment and constant phase models, respectively. The averages of these measurements for each mouse served as its baseline values. Following acquisition of baseline data, airway responsiveness to aerosolized methacholine (0 to 25 mg/ml saline; delivered by ultrasonic nebulizer) or serotonin (0 to 20 mg/ml saline) was assessed. Aerosols were delivered for 10 seconds without altering the ventilatory pattern, after which the 8 second forced oscillation perturbation was applied every 30 seconds for 5 minutes. Peak responses during each 5 minute period were determined for R, E, R_n, G and H. Linear interpolation was used to determine the provocative concentration of methacholine aerosol at which a 200% increase (PC200) over baseline values was observed for R, R_n and G and at which a 50% increase (PC50) over baseline values was observed for E and H. Brief occlusion of the expiratory tube was performed prior to each aerosol administration in order to reset the volume history. Only derived values with a coefficient of determination of 0.9 or greater were used.

Isometric force measurements in isolated bronchial rings. Mice were anesthetized with methoxyflurane followed by pentobarbital sodium (0.5-1.0 mg i.p.). The hearts and lungs were removed *en bloc*, and the left and right mainstem bronchial rings (~1-2 mm in diameter) were dissected free in ice-cold phosphate buffered saline solution (11). Rings were mounted on two tungsten wires, one connected to a fixed holder and the other to a force displacement transducer (Model FT03, Gould Electronics) for continuous measurement of isometric tension. The rings were immersed in pH-adjusted, oxygenated physiologic salt solution at 37°C. Tension data were relayed from transducers to a signal amplifier (600 series eight channel amplifier,

Gould Electronics). Data were acquired and analyzed using CODAS software (DataQ Instruments, Inc.). Rings were loaded with 0.1 g passive tension and then equilibrated for an additional 30 minutes before the studies began. The tension response of rings to incrementally increasing concentrations of carbachol (10^{-7} M to 10^{-3} M) was examined.

Immunoblotting. Lungs were homogenized in Tris Buffer (100 mM; pH 8.5) and protein concentrations of the homogenates were determined using the Bio Rad Assay reagent (Bio-Rad). Aliquots containing equivalent amounts of protein were prepared and boiled for 5 minutes in sample loading buffer containing sodium dodecyl sulfate (SDS). Twenty μ g of protein from each sample was subjected to electrophoresis on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked for one hour at room temperature in TBSTM (Tris buffered saline containing 0.1% Tween and 10% non-fat milk protein) and incubated with rabbit anti-mAChR M2 (Santa Cruz Biotechnology) diluted 1:100 in TBSTM at 4°C overnight. After removing primary antibody with several washes of TBST (Tris buffered saline with 0.1% Tween), the membrane was incubated with bovine anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 30 minutes. The membrane was washed as before and the antibody-antigen complexes were visualized using a chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The blot was stripped using Restore Western Blot Stripping Buffer (Pierce) and re-probed with goat anti-actin, an antibody reactive with a broad range of actin isoforms (Santa Cruz Biotechnology) and developed as before. Density of staining was assessed with the image analysis program Scion Image for windows (Scion Corporation) and M2 muscarinic receptor expression was normalized to actin expression by analyzing the M2 receptor/actin band density ratio in each sample.

Assessment of M2 muscarinic receptor function by electrical field stimulation.

Airway responsiveness to electrical field stimulation was assessed ex vivo as previously described (12), with some modifications. Briefly, segments of tracheas (3-4 mm in length) were isolated, cleaned of loose connective tissue and mounted in 3 ml organ baths (Harvard Apparatus Co. Inc) containing modified Krebs-Henseleit solution (in mM: 118 NaCl, 25 NaHCO₃, 2.8 CaCl₂·2 H₂O, 1.17 MgSO₄, 4.7 KCl, 1.2 KH₂PO₄, and 2 gm/l dextrose). Each tissue segment was supported longitudinally by two triangular stainless steel wire supports, inserted from the luminal side of the trachea. The lower support was attached to a stainless steel hook at the base of the organ bath, and the upper support was attached with a gold chain to an isometric force transducer (model FT.03C, Grass Instrument Co.), connected to P122 amplifiers (Grass-Telefactor Products). The transducer was mounted on a rack and pinion clamp permitting adjustments of the resting length of each tracheal segment. The baths were aerated with a 95% O₂-5% CO₂ gas mixture, and a pH of 7.43 ± 0.03 was established for the duration of each experiment. The temperature of the baths was maintained at 37°C. Each trachea segment was equilibrated in the bath for 60 minutes at an optimal resting tension of 1.0 gram. During this equilibration time, the tissues were challenged 3 times with KCl at a final concentration in the bath of 120 mM. Tracheas that would not develop a contractile response to KCl were excluded from further studies. Tissues were then rinsed with fresh Krebs-Henseleit solution and allowed to relax to their initial tension. To test M2 muscarinic receptor function, tissues were subjected to electrical field stimulation in presence or absence of the M2 muscarinic receptor antagonist gallamine (30 μ M), added 20 minutes before stimulation. Electrical field stimulation was delivered at increasing frequencies (1 to 30 Hz, 1 msec duration) using a Grass S44 stimulator connected to a Med-Lab Stimusplitter II. The stimulus was applied transmurally across the

tissues by means of parallel platinum electrodes (each 0.3 cm²), followed by recording of contractile responses using LabVIEW™ software.

Acetylcholine release assay. Acetylcholine (ACh) release was determined by spectrofluorometry using the Amplex® Red acetylcholine/acetylcholinesterase assay kit following the manufacturer's instructions (Molecular Probes). Briefly, individual mouse tracheas were placed in 37°C temperature-controlled organ baths containing 3 ml of Krebs-Henselheit buffer and subjected to electrical field stimulation (10 Hz for 1 min at 50 volts output) as described above. The solution was recovered from each bath and placed in tubes containing the acetylcholinesterase inhibitor physostigmine (10 µM). The solution was then subjected to two rounds of extraction with an equal volume of chloroform to remove physostigmine before the assays. The tracheal tissues were blotted on Whatmann paper and weighed. Data are expressed as pmol ACh released per min per mg tissue.

Allergic airway disease model. On Days 0 and 1, mice received an i.p. injection of 20 µg of ovalbumin (Grade V; Sigma) emulsified in 0.2 ml aluminum hydroxide adjuvant (Alhydrogel; Accurate Chemical and Scientific Corporation). Control mice received an i.p. injection of saline emulsified in 0.2 ml aluminum hydroxide adjuvant. On 5 consecutive days (Days 14–18), mice were challenged via the airways in a nose-only exposure chamber, for 30 min per day with 1% ovalbumin in saline aerosol. On day 19, invasive lung function measurements were performed as described above in “Respiratory Mechanics”. After assessment of lung function, lungs were lavaged with two 1 ml aliquots of HBSS, which were subsequently combined. Approximately 90% of the total injected volume was consistently recovered. The BAL fluid was centrifuged at $360 \times g$ for 10 minutes at 4°C. Aliquots of BAL fluid supernatants were stored at –80°C for total protein and cytokine analysis. BAL fluid cells

were resuspended in 1 ml HBSS and counted (Coulter Electronics Ltd). Slides of BAL fluid cells were prepared (Cytospin 3; Shandon), stained with Wright-Giemsa (Fisher Scientific), and differentiated using conventional morphological criteria in a blinded fashion. BAL fluid cytokines were analyzed using a Bioplex cytokine kit with the Bioplex Protein Array system (Bio-Rad) according to the manufacturer's instructions. Total protein in BAL fluid supernatants was assessed per the manufacturers' protocols, using the Bio-Rad protein assay (Bio-Rad).

Lung Histopathology. Lungs were inflation-fixed with 4% paraformaldehyde overnight, processed routinely, embedded in paraffin, sectioned (5 μ m) and stained with hematoxylin and eosin. A semi-quantitative histopathologic scoring system was developed and used to analyze the sections as follows: perivascular inflammation (0-3 points), peribronchial inflammation (0-3 points), giant cells (0-3 points), intraalveolar inflammatory micronodules (0-3 points), intraalveolar exudate (0-3 points), pleural inflammation (0-3 points). A total inflammatory score (range 0-18) was taken as the sum of individual scores. Scoring was performed by a pathologist who was blinded to genotype and treatment.

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Figure Legends

Figure E1. (A) Representative lung sections stained with H & E from allergic and non-allergic female wild type and α ERKO mice. (B) Lung inflammatory scores in lung sections from allergic and non-allergic female wild type and α ERKO mice. Data represent the means $\pm$ SEM of six non-allergic and fourteen allergic mice per group.

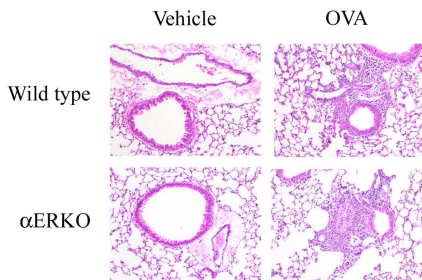
Table E1. Basal lung function in ovariectomized wild type and α ERKO mice

	Wild type	αERKO
	female ovex	female ovex
Number of animals	8	8
Body weight (g)	23.6 $\pm$ 1.0	22.9 $\pm$ 0.7
Penh	0.59 $\pm$ 0.02	0.60 $\pm$ 0.01
Frequency (breaths/min)	468 $\pm$ 8	432 $\pm$ 21
Tidal volume (μ l/breath)	306 $\pm$ 29	293 $\pm$ 17
Minute ventilation (μ l/min)	141 $\pm$ 14	125 $\pm$ 10
Peak inspiratory flow (ml/sec)	10.6 $\pm$ 1.1	9.8 $\pm$ 0.7
Peak expiratory flow (ml/sec)	7.0 $\pm$ 0.7	6.3 $\pm$ 0.5

Respiratory parameters were measured by non-invasive whole body barometric plethysmography in female wild type ovex and female α ERKO ovex. Data represent means $\pm$ SEM

Figure E1

A



B

