Male Sex Hormones Exacerbate Lung Function Impairment after Bleomycin-Induced Pulmonary Fibrosis

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The roles of sex hormones as modulators of lung function and disease have received significant attention as differential sex responses to various lung insults have been recently reported. The present study used a bleomycin-induced pulmonary fibrosis model in C57BL/6 mice to examine potential sex differences in physiological and pathological outcomes. Endpoints measured included invasive lung function assessment, immunological response, lung collagen deposition, and a quantitative histological analysis of pulmonary fibrosis. Male mice had significantly higher basal static lung compliance than female mice ($P < 0.05$) and a more pronounced decline in static compliance after bleomycin administration when expressed as overall change or percentage of baseline change ($P < 0.05$). In contrast, there were no significant differences between the sexes in immune cell infiltration into the lung or in total lung collagen content after bleomycin. Total lung histopathology scores measured using the Ashcroft method did not differ between the sexes, while a quantitative histopathology scoring system designed to determine where within the lung the fibrosis occurred indicated a tendency toward more fibrosis immediately adjacent to airways in bleomycin-treated male versus female mice. Furthermore, castrated male mice exhibited a female-like response to bleomycin while female mice given exogenous androgen exhibited a male-like response. These data indicate that androgens play an exacerbating role in decreased lung function after bleomycin administration, and traditional measures of fibrosis may miss critical differences in lung function between the sexes. Sex differences should be carefully considered when designing and interpreting experimental models of pulmonary fibrosis in mice.

Keywords: fibrosis; bleomycin; sex; respiratory mechanics

Forty years after its discovery (1), bleomycin is still an important, clinically relevant anti-neoplastic agent used as first-line therapy in the management of many human cancers, including Hodgkin's disease, germ cell tumors, and others (2–4). However, bleomycin induces pulmonary fibrosis in a dose-dependent manner, and fibrosis is a major dose-limiting side effect in patients receiving bleomycin (5). Other deleterious pulmonary side effects of bleomycin also limit its clinical utility, with observations of bleomycin-induced pneumonitis occurring as late as 2 years after cessation of bleomycin treatment (6).

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CLINICAL RELEVANCE

We describe a sex discrepancy in bleomycin-induced fibrosis, and illustrate the utility of lung function as an endpoint. This will facilitate more accurate modeling of fibrosis, and will provide a physiological endpoint to assess intervention efficacy.

The fibrosis-inducing side effect associated with bleomycin therapy observed in the human population has been exploited by researchers attempting to develop murine models of human interstitial pneumonias (7). These diffuse parenchymal lung disorders cause significant morbidity and mortality in the developed world (8) and present challenges to both the clinician and the researcher. Idiopathic pulmonary fibrosis (IPF) is one form of debilitating interstitial pneumonia with high morbidity and no available cure (9). The use of bleomycin and other fibrosis-inducing compounds in murine models has elucidated a number of signaling pathways associated with IPF; however, the translation from successful experimental models to efficacious clinical therapy has proved frustrating.

The experimental endpoints most frequently assessed in the murine bleomycin model include histological scoring of sectioned lung tissue, analysis of total lung collagen content, and infiltration of the lung by inflammatory cells. However, recent studies have demonstrated the utility of invasive lung function analysis in the murine bleomycin model of pulmonary fibrosis (10, 11) and suggest that the decline in lung function associated with fibrosis may not directly correlate with the traditionally assessed experimental endpoints. This observation may provide an explanation as to why previous experimental findings have been slow to generate useful clinical therapies, and provides researchers with a new experimental tool to use in searching for treatments of fibrosis and associated lung function decline.

Recent studies suggest that sex is an important factor in determining risk and prognosis for IPF, with males being more susceptible (12, 13) than their female counterparts. Numerous other studies indicate that sex is an important factor in other pulmonary pathologies, including asthma (14) and chronic obstructive pulmonary disease (15). The sex hormones are primary modulators of sex-specific physiology, and α and β estrogen receptor knockout (αERKO and βERKO) mice have been used extensively to elucidate that physiology in murine models (16–18). These findings, combined with the recent inclusion of lung function analysis as an experimental endpoint in pulmonary fibrosis models, raise a number of important questions and represent a new avenue of research readily amenable to study.

To assess the role of sex hormones in modulating bleomycin-induced pulmonary fibrosis in the murine model, the present study examined genetically and surgically altered C57BL/6 mice and used invasive assessment of respiratory mechanics after

bleomycin administration. Exogenous hormones were also administered to both surgically altered mice and intact mice. The results herein indicate that male sex hormones exacerbate the decline in lung function associated with pulmonary fibrosis, and that invasive analysis of respiratory mechanics may be a unique experimental endpoint that can reveal sex differences that are not obvious in other commonly used assays. Together, these findings indicate a need to stratify experimental groups based on sex, and provide further support for the use of invasive lung function analysis in fibrosis models.

MATERIALS AND METHODS

Animal Care and Treatments

All studies were conducted in accordance with principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences. Wild-type male and female C57BL/6 mice (10–20 wk old) were purchased from Taconic Farms (Rockville, MD). αERKO and βERKO mice on a C57BL/6 background (8–12 wk old) were also purchased from Taconic. When performed, surgical castration of male and ovariectomy of female mice occurred 3 weeks before administration of bleomycin. All gonadectomies were performed on mice at least 8 weeks old. Subcutaneous implantation of pellets containing 5-α-dihydrotestosterone (DHT, 15 mg, 60 d release; Innovative Research of America, Sarasota, FL) or placebo into castrated male mice or intact female mice was performed concomitantly with gonadectomy or 3 weeks before experimentation in animals that were not surgically altered.

For bleomycin treatments, mice between the ages of 10 and 20 weeks were anesthetized with isoflurane/oxygen and were administered bleomycin sulfate (B5507; Sigma-Aldrich, St. Louis, MO) dissolved in sterile, endotoxin-free saline (S8776; Sigma-Aldrich), or an equivalent volume of sterile saline (up to 75 μl) as a control via oropharyngeal aspiration as described previously (10). Briefly, mice were anesthetized and placed on a plastic dosing board suspended by their upper incisors on a piece of suture strung between two pegs. The tongue was extended with forceps to prevent the mouse from swallowing, and either drug or vehicle was pipetted into the back of the throat. A brief occlusion of the nose forced the mouse to inhale orally, aspirating the solution into the lungs in one or two quick breaths. Mice were returned to their cages, where they rapidly regained consciousness and displayed no immediate ill effects from the dosing.

Doses of bleomycin were body weight adjusted to either 1 mg/kg or 2 mg/kg. These doses correspond to approximately 1.5 Units/kg and 3 Units/kg, meaning that a 30-g mouse received approximately 0.045 Units or 0.090 Units of bleomycin, respectively. The 1 mg/kg dose was used throughout the study unless explicitly stated otherwise. After bleomycin administration, mice were observed daily for signs of morbidity and weighed on Days 3, 7, 10, and 14. Mice were fed mashed food to help prevent excess weight loss, and all lung function assessments and tissue collections were performed 21 days after dosing unless explicitly stated otherwise.

Invasive Assessment of Respiratory Mechanics

Invasive assessment of lung function was performed as previously detailed (10). All lung function analysis was performed using the Flexivent system (SCIREQ, Montreal, PQ, Canada). Mice were anesthetized (urethane) and paralyzed (pancuronium bromide), and a portable heart rate monitor (CardioMonitor; BAS Vetronics, West Lafayette, IN) was used to ensure that appropriate anesthesia was maintained throughout the duration of ventilation. Ventilation was maintained at a rate of 150 breaths/minute, a tidal volume of 7.5 ml/kg, and a positive end expiratory pressure of approximately 3 cm of water. Mice were allowed to acclimate to the ventilator for 2 to 3 minutes before the initiation of readings, and three to four total lung capacity functions were performed during this acclimation period to prevent atelectasis and to ensure maximum airway and alveolar recruitment.

Pressure–volume curves were generated and static compliance (C_{st}) was calculated by the Flexivent software (version 5.1) using the Salzar-Knowles equation (19). Thirty seconds after each pressure–volume curve, a 2-second perturbation at 2.5 Hz was applied to generate data using the single compartment model of respiratory mechanics. Total respiratory system elastance (E) was calculated from this data by the Flexivent software, and from elastance, dynamic compliance (C) was determined. After pressure–volume curve data collection, the mouse was again rested for 30 seconds and then an 8-second pseudorandom perturbation consisting of waveforms of mutually prime frequencies (0.5–19.6 Hz) was performed. Data generated from these perturbations were fit to the constant phase model of respiratory mechanics, and tissue elastance (H) was calculated by the Flexivent software. Repeated measurements were taken per subject and only data with a coefficient of determination greater than 0.95 were included in the final analysis. At least three pressure–volume loops, three single compartment perturbations, and three constant phase perturbations, all with acceptable coefficients of determination, were recorded per animal. The average of these measurements was determined on a per-mouse basis, and the mean and standard error of those averages is reported for each experimental group.

Assessment of Airway Inflammation and Tissue Collection

Bronchoalveolar lavage fluid (BALF) was collected from mice immediately after lung function assessment. A total volume of 2.0 ml (2 × 1 ml) of Hanks' Balanced Salt Solution (H6648; Sigma-Aldrich) was instilled into the lungs and 80 to 90% was routinely recovered. Recovered BALF was processed and analyzed for total cell counts and differentials, and aliquots of cell-free BALF were archived at −80°C. The right caudal lobe of the lungs was tied off and extracted for collagen content, and the remaining right lung lobes archived at −80°C. Left lung lobes were inflated and fixed with 4% paraformaldehyde for preparation of histology slides.

Collagen Assay

The right caudal lobe was homogenized in RIPA buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) plus protease inhibitors using an Ultra Turrax homogenizer. (Ultra Turrax 25; IKA, Staufen, Germany). Total collagen content was subsequently determined using the colorimetric Sircol Collagen assay kit (Biocolor, Carrickfergus, United Kingdom; distributed in the United States by Accurate Chemical, Westbury, NY) according to manufacturer's instructions. Results are expressed as micrograms collagen per lung lobe.

Real-Time PCR Analysis

Total RNA was purified from lung lysates using Qiagen RNeasy Midiprep kits according to manufacturer's instructions (Qiagen, Valencia, CA) including optional treatment with Qiagen RNase-free DNase (Qiagen cat. # 79,254). RNA was then reverse transcribed into first-strand cDNA using the ABI/Prism High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). All PCR reactions were performed in triplicate as follows: 2 minutes at 50°C, 10 minutes at 95°C, then 35 cycles each at 95°C for 15 seconds followed by 60°C for 60 seconds in the ABI/Prism 7900 HT Sequence Detector System. For bleomycin hydrolase, probe #Mm00724434_m1 from Applied Biosystems was used. All results were normalized to an internal control transcript encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) using probe # Mm99999915_g1. Lungs from at least four different animals per group were used.

Histology

Histological assessment of the extent and severity of pulmonary fibrosis was independently determined by two blinded pathologists using the Ashcroft method (20) on sections of the left lung (5–6 μm) stained with Masson's trichrome. A more detailed assessment of lung fibrosis was also performed as previously described (10), again by a pathologist blinded to sex and treatment group. This scoring system was designed to specifically identify fibrosis associated with small to medium-sized airways. Fibrosis in these airways was determined by quantifying subepithelial collagen thickness around bronchioles with an internal diameter of 100 to 200 μm. Bronchioles that appeared as a closed circular profile were selected from Masson's-stained slides, and photomicrographs of five bronchioles per individual animal were digitized with a ×10 objective from a Zeiss Axioskop 2 microscope (Carl Zeiss, Thorwood, NY) accompanied by the

SPOT advanced digital image analysis program (Diagnostic Instruments Inc, Sterling Heights, MI). Measurements were initiated by centering an eight-spoke wheel in the middle of the digitized image of each bronchiole using the Freehand MX software program (Macromedia, San Francisco, CA). The thickness of the collagen layer immediately adjacent to airway walls at each point of intersection between the wheel and the bronchiole was determined using a digital ruler available in Adobe Photoshop version 8.0 (Adobe Systems, San Jose, CA). An average for each airway was determined based on these measurements, and individual intersections were excluded from analysis if they were associated with a region of airway wall immediately adjacent to a vessel with apparent perivascular collagen. This allowed the pathologist to score for the collagen associated with airways while avoiding confounding adjacent vessels. An average for each individual animal was calculated based on at least five independently analyzed airways. Typically, six to seven intersections were scored per bronchiole, at least five bronchioles scored per animal, and at least five animals per treatment group were included in the analysis.

Statistical Analysis

All data are expressed as group means ± SEM. One-way ANOVA followed by Newman-Keuls Multiple Comparison Test was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). In all instances, statistical significance is defined as $P < 0.05$ compared with the indicated group.

RESULTS

Static Compliance Decreases in Male but Not Female Mice after Bleomycin Exposure

Bleomycin administered via oropharyngeal aspiration to anesthetized C57BL/6 mice resulted in a decrease of approximately 5% body weight 1 week after dosing. All mice in the bleomycin groups were provided mashed food to minimize weight loss, and all mice in the 1 mg/kg group returned to pre-dosing body weight on or around Day 14 after dosing. At 2 mg/kg, approximately 10% of the bleomycin-dosed animals were killed before the end of the experiment due to excessive weight loss and morbidity. Saline-treated animals displayed no ill effects and no weight loss throughout the 21 days of study.

In male mice, 1 mg/kg of bleomycin resulted in a significant decrease in quasi-C_{st}, a measure of the elastic recoil pressure of the lungs at a given volume (Figure 1), compared with saline-treated mice. Similar results were obtained for dynamic compliance (C) using the single compartment model of respiratory mechanics (data not shown). Dynamic compliance reflects the ease with which the lungs can be expanded at normal respiratory rates, while static compliance describes a similar condition at a given volume. These results indicate a dose-dependent decline in lung function as well as dose-dependent mortality in male C57BL/6 after bleomycin administration.

In age-matched female C57BL/6 mice, no bleomycin-induced decline in lung function was observed after either the 1 mg/kg or the 2 mg/kg dose (Figure 1). At 2 mg/kg, a similar amount of mortality was observed in the female mice compared with the male mice between 10 and 15 days after dosing. However, the studies were not designed with survival as an endpoint, and as such the data is not amenable to statistical analysis. A significant difference in C_{st} was observed between saline-treated male and female animals. However, this difference in baseline did not account for the observed male/female differences after bleomycin dosing, as results remained significant whether expressed as a whole number or as a percentage of baseline. Naïve, untreated male ($n = 14$) and female ($n = 11$) mice also displayed a statistically significant sex difference ($P < 0.01$) in baseline C_{st} that was similar to that observed in saline-treated mice (data not shown). The 2 mg/kg bleomycin dose resulted in a greater than 50% decrease in C_{st} compared with saline controls in male mice

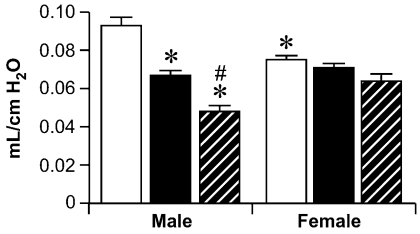

Figure 1. Static compliance decreases in bleomycin-treated male mice in a dose-dependent manner while female lung function remains unaltered. Quasi-static compliance (C_{st}) was measured via Flexivent 21 days after bleomycin administration at the indicated doses. Data presented represent pooled readings from three separate experiments (n = 12, 18, 8, 11, 20, and 8, respectively; $^{*}P < 0.05$ compared with male saline; $^{\#}P < 0.05$ compared with male 1 mg/kg). *Open bars*, saline; *solid bars*, 1 mg/kg; *hatched bars*, 2 mg/kg.

(0.0485 ml/cm H_2O versus 0.0931 ml/cm H_2O, respectively) while the same dose in female mice resulted in an approximately 10% decrease in C_{st} compared with saline controls (0.0645 ml/cm H_2O versus 0.0752 ml/cm H_2O, respectively). These data suggest that male mice are more susceptible than female mice to bleomycin-induced lung function decline.

Commonly Measured Parameters of Lung Fibrosis Are Similar in Male and Female Mice

Instillation of bleomycin induces a number of well-characterized cellular, biochemical, and morphological changes in the lung (reviewed in Ref. 21), including infiltration of inflammatory cells, increased collagen deposition, and quantifiable morphological changes in the lung architecture (20). Considering the observed differences in lung function, we analyzed the data collected from these commonly reported endpoints according to sex. Because of the clear differences observed in lung function at 1 mg/kg and the increased mortality of mice at 2 mg/kg bleomycin, all subsequent experiments were performed with the 1 mg/kg dose. This dose of bleomycin resulted in a significant influx of inflammatory cells into the BALF of male and female mice, with the largest single constituent being lymphocytes (Figure 2A). At both baseline and after bleomycin administration, immune cell presence in the lungs of male and female mice was similar, suggesting that the underlying cause of the observed differences in lung function is independent of immune cell presence 21 days after bleomycin exposure. Total lung collagen deposition was similarly increased in both males and females (Figure 2B), with no significant difference between the sexes. Histopathological changes typical of bleomycin exposure were observed in sectioned lung tissue from both males and females (data not shown), and the results were analyzed in a quantifiable manner (Figure 2C). Similar to cellular infiltration and collagen deposition, significant changes in both males and females were observed after bleomycin administration, but no difference was observed between the sexes. Together these data indicate that several commonly reported endpoints associated with pulmonary fibrosis are insufficient to explain the observed differences in lung function after bleomycin administration.

Bleomycin Hydrolase Levels Do Not Differ between the Sexes at Early or Late Time Points

Detoxification of bleomycin occurs *in vivo* as a result of hydrolysis by the enzyme bleomycin hydrolase (22, 23). Heterogeneity in expression and/or activity of bleomycin hydrolase has long been suspected as contributing to pulmonary fibrosis (24), and more

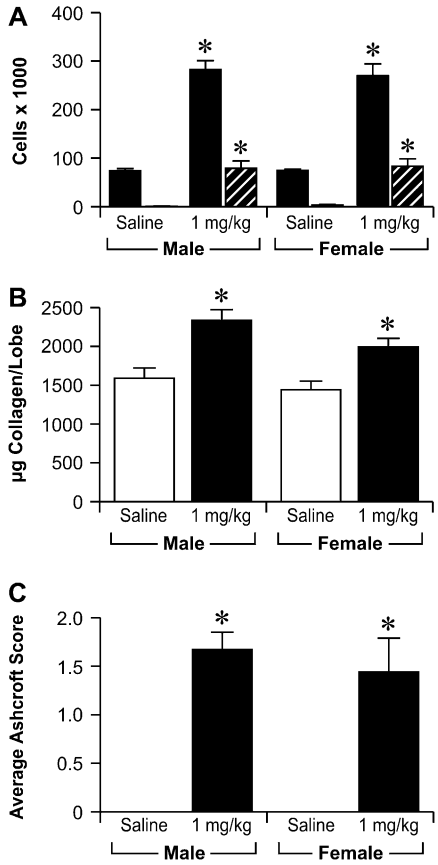

Figure 2. Bleomycin induces similar cellular, biochemical, and histological changes in male and female mice. (*A*) Bronchoalveolar lavage fluid cells in saline- and bleomycin-treated mice. *Solid bars*, total cells; *hatched bars*, lymphocytes. (*B*) Total lung collagen content as assessed by Sircol assay. (*C*) Fibrosis scores calculated on the basis of histological assessment of trichrome-stained lung sections. For *A*, *B*, and *C*, data presented are representative examples of three separate experiments and contain the same subjects for which lung function data were collected ($n = 6$, 10, 6, and 10, respectively; $^*P < 0.05$ compared with same-sex saline controls). Male and female saline-treated groups both have Ashcroft scores of zero and are represented by *blank space* in the *bar graph*. There was no significant difference in males compared with females at either baseline or after bleomycin administration in any of the endpoints.

recent studies have suggested that differential activity of bleomycin hydrolase may occur between the sexes (25). Consistent with the notion that bleomycin hydrolase activity is up-regulated after bleomycin administration, we observed an increase in bleomycin hydrolase mRNA after bleomycin administration (data not shown). However, similar to the cellular infiltration and collagen deposition data, no significant difference in bleomycin hydrolase expression levels was observed between males and females at baseline or after bleomycin exposure either 3 or 21 days after bleomycin administration. When normalized to GAPDH, male ΔCt at 3 days after bleomycin treatment was 4.23 $\pm$ 0.25 compared with that of females (4.36 $\pm$ 0.23). At 21 days, male ΔCt was 4.12 $\pm$ 0.24 compared with female ΔCt of 4.10 $\pm$ 0.62.

Regional Differences in Fibrosis May Contribute to Observed Differences in Lung Function

While quantitative whole lung histology failed to reveal any differences between male and female mice after bleomycin administration (Figure 2C), regional differences in collagen deposition could contribute to the observed lung function disparity. To test this hypothesis, we employed a previously described method designed specifically to quantify subepithelial collagen deposition associated with intermediate-sized airways (10). Representative airways are illustrated in Figure 3A, and the reported Ashcroft score assigned to the whole lung by the pathologist is provided as a basis for comparison. The single airway score refers to the regional score assigned as described in MATERIALS AND METHODS for the pictured airway. The total airway score refers to the average of at least five separate airways for the illustrated animal. As indicated in Figure 3B, bleomycin-induced collagen deposition and fibrosis adjacent to intermediate-sized airways in a manner similar to that observed for the whole lung. An apparent difference was observed between the airway

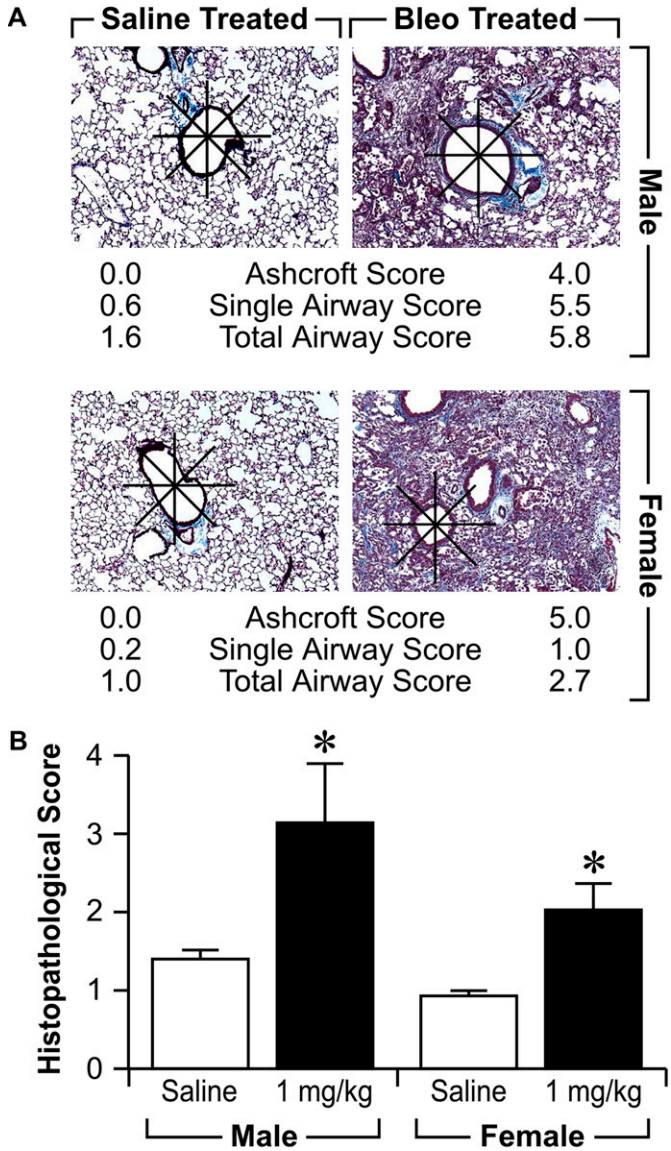

Figure 3. Assessment of airway-specific fibrosis reveals a trend toward more collagen adjacent to small and intermediate-sized airways in male mice. (*A*) Photomicrographs of representative airways selected at random for analysis of subepithelial collagen thickness. (*B*) A trend toward more collagen deposition immediately adjacent to airways in male mice was observed ($P = 0.12$). $n = 5$ for saline groups and $n = 8$ for bleomycin groups; $^*P < 0.05$ compared with same-sex saline controls.

fibrosis scores in bleomycin-treated males and females, although this difference did not reach statistical significance (Figure 3B). As such, while this mechanism alone is unlikely to account for the observed differences in lung function between the sexes, it may be at least partially involved.

Estrogen Does Not Mediate Bleomycin-Induced Lung Function Decline

Considering the critical role of sex hormones in modulating the physiological and pathological differences in male and female lungs (26, 27), we first assessed lung function in male and female α and βERKO mice. As illustrated in Figure 4A, female αERKO and βERKO mice responded to bleomycin in a manner virtually identical to that of their female wild-type counterparts. Figure 4B reveals that male mice were similarly indifferent to the presence of functional estrogen receptors. Thus, male α- and βERKO mice had a similar decline in C_{st} compared with their male wild-type counterparts. Other endpoints analyzed (cell counts, differentials, collagen deposition) remained similarly unchanged in α- and βERKO mice (data not shown). These data indicate that either estrogen plays an insignificant role in modulating the lung response to bleomycin, or that the two isoforms of the estrogen receptor are redundant and can both provide the necessary downstream signaling required to protect the female lung from a decline in lung function after bleomycin exposure. To differentiate between these two possibilities, circulating estrogen was removed by ovariectomizing female mice. As illustrated in Figure 4C, ovariectomy 3 weeks before bleomycin administration had no impact whatsoever on the female response to bleomycin, with ovariectomized mice having similar C_{st} at baseline and after bleomycin exposure. Together, these data strongly suggest that in the murine model of bleomycin-induced pulmonary fibrosis, lung function declines independent of estrogen in both male and female mice.

Androgen Exacerbates Bleomycin-Induced Lung Function Decline in Male and Female Mice

Given that estrogen did not provide a protective benefit to female mice, we hypothesized that androgen may have exacerbated the response to bleomycin in this model, consistent with previous observations in a different model of lung disease (28). To test this hypothesis, lung function analysis was performed in castrated males as well as castrated males treated with exogenous DHT. As illustrated in Figure 5A, castration of male mice attenuated the decrease in C_{st} observed after bleomycin exposure, lowering the baseline while simultaneously making the mice more resistant to the effects of bleomycin. Comparing Figure 5A castrated male saline and 1 mg/kg to Figure 1 female saline and 1 mg/kg, it is evident that castration feminized male C_{st} at baseline as well as in response to bleomycin. Furthermore, addition of exogenous DHT at the time of castration completely prevented this feminization, restoring the statistical significance eliminated by castration. As illustrated in Figure 5B, the deleterious effects of androgen in this model were not limited to male mice. Treatment of female mice with DHT for 21 days before bleomycin exposure resulted in a statistically significant decrease in C_{st}, masculinizing the female response to bleomycin. It is important to note that throughout these experiments, the only statistically significant

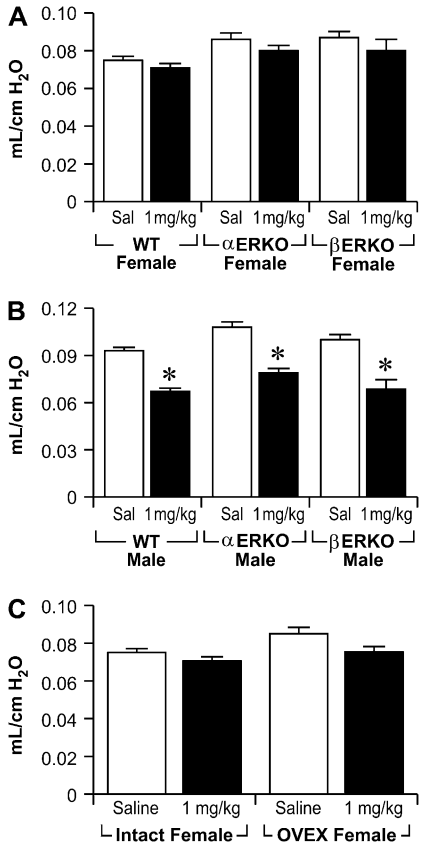

Figure 4. Estrogen does not protect female mice from decrease in lung function after bleomycin administration. (*A*) Quasi-static compliance in female mice is similar among wild-type (WT), α-ERKO, and β-ERKO groups (n = 11, 20, 6, 22, 6, and 11, respectively). (*B*) Quasi-static compliance in male mice is similar among wild-type, α-ERKO, and β-ERKO groups (n = 12, 18, 6, 10, 6, and 10, respectively; *$P < 0.05$ compared with the corresponding group saline control). (*C*) Quasi-static compliance in similar among intact and ovariectomized females (n = 11, 20, 4, and 8, respectively).

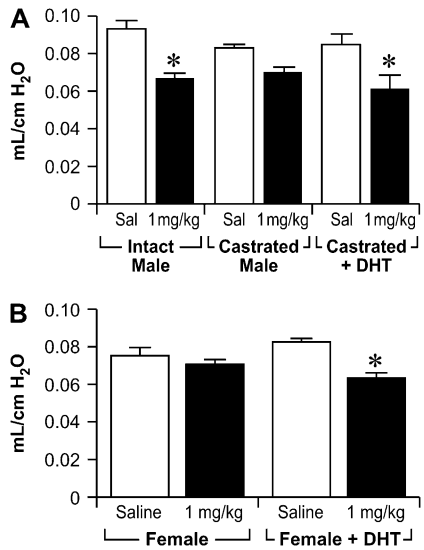

Figure 5. Androgen exacerbates decline in lung function in males and females. (*A*) The decrease in baseline quasi-static compliance observed in male mice after castration is partially restored by addition of exogenous DHT. The decrease in quasi-static compliance in male mice after bleomycin is attenuated after castration and restored by exogenous DHT (n = 12, 18, 4, 8, 4, and 8, respectively). (*B*) Quasi-static compliance measured in intact female mice and DHT-treated intact female mice (n = 11, 20, 4, and 8, respectively). *$P < 0.05$ compared with the corresponding saline control in both *A* and *B*.

decrease in female C_{st} in response to bleomycin exposure occurred in the presence of androgen.

Together, these data support a causative role for androgen in leading to a more severe decline in lung function after bleomycin exposure. Considering the previously described trends in collagen deposition associated with airways, we analyzed our androgen-treated population to see if castration and/or exogenous androgen administration changed either the Ashcroft score or the airway-specific collagen deposition. Histology was performed on slides prepared from castrated males ($n = 8$), DHT-treated castrated males ($n = 8$), and DHT-treated females ($n = 8$). Neither the Ashcroft scores nor the airway-specific collagen deposition was altered in a statistically significant manner in any of the treatment groups (data not shown).

DISCUSSION

This study was designed to elucidate the contributions of sex and sex hormones to the progression of lung fibrosis and associated lung function decline in a murine model. To this end, the established and well-characterized bleomycin model was used to induce pulmonary fibrosis in reproductive-age male and female C57BL/6 mice. Fibrosis was assessed with common biochemical and histological endpoints, and invasive lung function analysis was employed to measure respiratory mechanics. Our data suggest that lung function analysis is a unique indicator of lung damage not captured by other more commonly used endpoints. Lung function decline also displays a sex specificity not observed in the other endpoints, a finding that may allow researchers to more accurately model human diseases in which sex differences exist. Furthermore, decreased lung function may not necessarily mirror immune cell influx, collagen deposition, or total lung fibrosis in the murine model. This is consistent with our previous observations in cyclooxygenase-2–deficient mice (10). Furthermore, we demonstrated an exacerbatory role for androgen in mediating the decline in lung function associated with fibrosis, but not the progression of fibrosis itself. By comparison, estrogen appeared to play no role in modulating lung function after bleomycin administration, as αERKO, βERKO, and ovariectomized female mice responded identically to intact wild-type females. Female mice responded to bleomycin with decreased lung function if and only if they had been treated with DHT, further illustrating the exacerbatory role of androgen in decreasing lung function after bleomycin administration.

The possibility of sexual disparity in the response to bleomycin in the murine model has been raised before (25) and is consistent with recently published findings in humans that describe sex differences in the severity of emphysema (29). However, our data are in contrast to recent findings suggesting that estrogen plays an important role in pulmonary fibrosis in a different species (30). We currently have no explanation for the differences in our study compared with that of Gharaee-Kermani and colleagues (30); however, it is important to note that our study was performed with mice while theirs was performed with rats, and that we have included invasive lung function analysis as an endpoint. Further work is required before we can say definitively why the dichotomy exists between the two rodent models.

Bleomycin hydrolase is a neutral cysteine protease of the papain superfamily (31) named for its ability to catalytically deaminate and inactive bleomycin (23). In humans, bleomycin hydrolase is widely expressed (32), although evidence exists to suggest that activity in lungs is heterogeneous (24) and may be decreased as a result of bleomycin administration (33). Bleomycin hydrolase–null mice display a profoundly increased sensitivity to bleomycin (34), suggesting that bleomycin hydrolase is the predominant, if not sole, bleomycin-detoxifying enzyme. Moreover, bleomycin hydrolase is a candidate gene for a recently identified sex-specific genetic locus conferring susceptibility to bleomycin-induced pulmonary fibrosis (25). In our studies, we observed no difference in bleomycin hydrolase levels between untreated wild-type male and female mice as assessed by real-time quantitative PCR. Furthermore, while exposure to bleomycin increased bleomycin hydrolase expression, there was no observed difference in expression between male and female mice. Based on these data, we think it unlikely that differential bleomycin hydrolase expression accounts for the observed differences in lung function. This conclusion is supported by the observed similarities in total lung collagen deposition and fibrotic response independent of the difference in lung function.

Lung function as an endpoint in the murine model of bleomycin-induced lung fibrosis is a relatively new and important development and, in our view, allows researchers to more accurately reflect the human pathological condition that the model was designed to emulate. Declining lung function more directly correlates with poor prognosis in humans with pulmonary fibrosis than do fibrotic endpoints (35) and may have more predictive value than the commonly reported collagen content, immune cell influx, and Ashcroft score (36). The importance of including lung function analysis as part of the murine model is underscored by our findings that there is a quantifiable and significant difference between males and females in their static and dynamic compliance after bleomycin despite having similar cellular and histological responses, and that androgen appears to impact lung function while not altering other commonly measured endpoints associated with bleomycin. Other studies that have included lung function in the bleomycin model lead to disparate conclusions regarding the correlation between compliance and fibrotic endpoints. We have previously reported discordance between the fibrotic endpoints and lung function (10), while others have reported an agreement between the compliance and fibrotic endpoints (11). It is worth noting that Lovgren and colleagues (11) used multiple strains of mice and administered a dose of bleomycin that was not adjusted for body weight. Indeed, the resulting doses of bleomycin administered by Lovgren and coworkers were higher than what we used for most of our experiments. It is possible that higher doses may obscure the point at which lung function and other endpoints diverge. We specifically selected a dose of bleomycin that was in the middle to low range of reported doses to maximize our ability to detect subtle changes or changes that may be masked by larger doses.

Although not the focus of this study, total respiratory system resistance (R) was observed to increase in bleomycin-treated male but not female mice (data not shown), and we believe this may have been at least partially due to the increased collagen deposition seen around the airways in males. Other studies did not demonstrate increased R after bleomycin, but they differed from the current study in that they used either female mice only (10) or a mix of sexes (11). The implications of this observation are currently unclear, but will be investigated in future studies. Furthermore, our findings are consistent with those of Lovgren and coworkers and others who have reported that administration of bleomycin results in a decrease in lung compliance. This is consistent over a number of mammalian models including mice, rats (37), and rabbits (38), and is consistent with findings in humans demonstrating decreased lung function in patients with fibrosis (36).

The mechanism by which androgen exacerbates the decrease in static and dynamic compliance in our model remains undetermined. Our data suggests that multiple mechanisms may be involved, as castration changes both baseline quasi-static compliance and the response of the animal to bleomycin (Figure 5A). Indeed, both of these changes are necessary to observe

statistically significant differences after castration. Moreover, addition of exogenous DHT concomitant with castration increases baseline C_{st} and normalizes the response to bleomycin. Furthermore, as illustrated in Figure 5B, addition of exogenous DHT similarly alters the response of both saline- and bleomycin-treated mice. It should be noted that the only statistically significant difference that was observed in quasi-static compliance in females was in the presence of DHT, and both the baseline and the bleomycin responses changed. A number of potential explanations exist and are the subject of ongoing experimentation. Injury to specific cell types within the lung and/or the immune system, changes in noncollagen components of the extracellular matrix, differences in collagen maturity, and microenvironmental differences in extracellular matrix changes are some of the potential mechanisms being investigated. Among these, we have examined regional differences in collagen deposition in this study. While our observations are consistent with fibrosis being a predominantly parenchymal phenomenon, there were differences between the sexes in the amount of collagen associated with small and intermediate-sized airways that tended to be greater in males than in females. These differences underscore the challenges involved in describing and quantifying the morphological changes associated with a condition as heterogeneous as pulmonary fibrosis while highlighting the utility and importance of including invasive lung function in studies of pulmonary fibrosis.

A number of recent studies have highlighted the myriad of ways in which sex hormones can modulate lung development, physiology, and pathology (reviewed in Refs. 26, 27). Sex hormones have been demonstrated to play important roles in models of allergic airway disease, immune response, and lung injury, and the influence of sex hormones is often dependent upon the model studied. In this article we have described a model in which male mice suffered a more severe decrease in lung compliance than female mice after bleomycin administration. Conversely, in a *Pseudomonas aeruginosa* infection model in the same C57BL/6 strain of mice, females are demonstrably more susceptible to infection and display a more robust immune response than males (39). Our study further showed that castration attenuated the observed differences while ovariectomy did nothing to modulate the phenotype, and that addition of exogenous androgen resulted in a return of castrated males to their pre-surgery response level as well as masculinizing the response of female mice. These data suggest that estrogen plays an insignificant role in protecting females from lung function decline after bleomycin exposure despite the mounting of a fibrotic response similar to that of males. Furthermore, these data indicate that androgen can exacerbate the lung function decline in males and females after bleomycin administration, a finding consistent with previously published data demonstrating an exacerbatory role for androgens in modulating airway responsiveness to methacholine during LPS-induced inflammation (40).

In closing, the findings described herein reveal an androgen-mediated sex difference in lung function decline in a murine model of pulmonary fibrosis that is not reflected in more common biochemical and histological measures of fibrosis. Our data indicates that more specialized histopathologic measurements of airways in the lung may be helpful to capture regional fibrotic responses. Furthermore, our data highlights the utility of invasive lung function analysis in experimental studies of pulmonary fibrosis and indicate the need to stratify experimental design and data analysis based on sex. Future investigations will focus on elucidating the mechanism(s) by which androgens promote lung function decline in pulmonary fibrosis with the expectation that such insights might lead to the development of novel approaches to combat this condition.

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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