Gender influences the response to experimental silica-induced lung fibrosis in mice

David M. Brass,1 Sean P. McGee,2 Mary K. Dunkel,3 Sarah M. Reilly,3 Jacob M. Tobolewski,3 Tara Sabo-Attwood,2 and Cheryl L. Fattman3

1Department of Pediatrics, Duke University, Durham, North Carolina; 2Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, South Carolina; and 3Department of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania

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Brass DM, McGee SP, Dunkel MK, Reilly SM, Tobolewski JM, Sabo-Attwood T, Fattman CL. Gender influences the response to experimental silica-induced lung fibrosis in mice. Am J Physiol Lung Cell Mol Physiol 299: L664–L671, 2010. First published August 20, 2010; doi:10.1152/ajplung.00389.2009.—Accumulating evidence suggests that gender can have a profound effect on incidence and severity of a variety of pulmonary diseases. To address the influence of gender on the development of silica-induced pulmonary fibrosis, we instilled 0.2 g/kg silica into male and female C57BL/6 mice and examined the fibrotic and inflammatory response at 14 days postexposure. Both silica-exposed male and female mice had significant increases in total lung hydroxyproline compared with saline controls. However, silica-exposed female mice had significantly less total lung hydroxyproline than silica-exposed male mice. This observation was confirmed by color thresholding image analysis. Interestingly, silica-exposed female mice had significantly more inflammatory cells, the majority of which were macrophages, as well as higher levels of the macrophage-specific chemokines MCP-1 and CCL9 in whole lung lavage compared with silica-exposed male mice. We also show that at baseline, estrogen receptor α (ERα) mRNA expression is lower in female mice than in males and that ERα mRNA expression is decreased by silica exposure. Finally, we show that the response of ovarioctomized female mice to silica instillation is similar to that of male mice. These observations together show that gender influences the lung response to silica.

In the developed world, increasingly stringent regulations have reduced occupational exposures to dusts that cause pneumoconiotic lung diseases such as PF (1). However, dust-related lung diseases resulting from occupational exposures represent a considerable health care cost burden in rapidly industrializing nations (11, 34, 37). Commonly, the highest exposures to occupational dusts are seen in the least-skilled occupations, which, in the developing world, are often filled by women (2, 11, 36, 47–49). Thus it is important to understand the biology of gender-based differences to occupational dust exposures.

To address the hypothesis that gender contributes to the response to occupational dust exposure, we exposed both male and female C57BL/6 mice to crystalline silica and examined the fibrotic and inflammatory responses. We report here for the first time that the response to silica-induced lung fibrosis is dependent on gender at the silica dose and time point examined.

MATERIALS AND METHODS

Animals. Eight-week-old C57BL/6 (Jackson Laboratory, Bar Harbor, ME) were given one intratracheal instillation of 0.2 g/kg crystalline silica (a generous gift from Dr. Andy Ghio, Environmental Protection Agency) in 60 μl of sterile 0.9% saline or an equivalent volume of 0.9% saline and then killed at 14 days posttreatment. Lungs were lavaged as previously described with 1.5 ml of 0.9% saline (21). All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Histological and morphometric analysis. Lung sections were stained with Mason’s trichrome to visualize collagen. The entire lung was photographed at ×20 magnification, and Nikon Elements software was used to calculate tissue volume density (TVD) defined as the percentage of each microscopic field that is lung tissue as well as the percentage of each tissue-containing field that is collagen. Tissue (red) and collagen (blue) staining areas were sampled using six micrographs for each lung as an internal control to account for variability in staining. Values for all fields were averaged to yield a single TVD per animal. TVD values per animal were then averaged to yield a group average.

Analysis of bronchoalveolar lavage fluid. Total protein in lavage fluid was determined by Coomassie blue protein assay (Pierce, Rockford, IL) according to the manufacturer’s instructions. A Beckman Z1 dual Coulter particle counter (Beckman Coulter, Fullerton, CA) was used to obtain total white blood cell counts. White blood cell differentials were determined by light microscopy of Diff Quik-stained (Dade Behring, Newark, DE) cytospin slides. A total of 400 cells/cytospin slide were counted, and the percentage of total cells that were macrophages and polymorphonuclear leukocytes (PMNs) was determined. Total MCP-1, KC, osteoprotegerin (OPG), and CCL9 concen-
trations in BALF were determined using a Duo-Set ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Hydroxyproline analysis. Whole lungs were lyophilized and acid hydrolyzed in sealed, oxygen-purged glass ampules containing 2 ml of 6 N HCl at 110°C for 24 h. Samples were resuspended in 1.5 ml of PBS and incubated at 60°C for 1 h. Samples were centrifuged at 13,000 rpm for 10 min, and hydroxyproline analysis was performed on the resulting supernatant using chloramine-T as previously described (53).

Estradiol ELISA. Concentrations of circulating estradiol were determined in serum from saline and silica-treated mice by ELISA (ALPCO Immunoassays, Salem, NH) according to the manufacturer’s instructions.

RT-PCR. Messenger RNA expression of the estrogen receptors (ER) and G protein estrogen receptor (GPER) in mouse lung tissues was performed essentially as previously described (42). Total RNA was isolated from whole lung tissue using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and was further purified using the RNeasy system (Qiagen, Valencia, CA). RNA concentration was determined by measuring the absorbance at 260 nm, and quality was assessed on a 2100 Bioanalyzer (Agilent, Palo Alto, CA). Total RNA (1 μg) was reverse-transcribed, and the resultant cDNA was amplified for each gene-specific target using standard TaqMan Universal PCR Master Mix and the following prevalidated Assays on Demand (Applied Biosystems). Expression assays for each gene were run in duplicate, normalized to the HPRT housekeeping gene (Mm01281854_m1; ERβ, Mm01281854_m1; GPER, Mm01194815_m1 (Applied Biosystems, Carlsbad, CA). All reactions were performed on a 7900 HT system (Applied Biosystems). Expression assays for each gene were run in duplicate, normalized to the HPRT housekeeping gene (Mm01281854_m1), and expressed as fold change over control calculated by the ΔΔCt method. Statistical analyses. All data are expressed as means ± SE. Primary comparisons were between fibrotic (total lung hydroxyproline) and inflammatory responses (total lavage cells, differential and cytokine responses) in male and female C57BL/6 mice at 14 days postsilica instillation. Differences between groups and between variables were analyzed by ANOVA. Probability values of P < 0.05 (2-tailed) were considered statistically significant.

RESULTS

Female C57BL/6 mice are partially protected from silica-induced fibrosis. To address the role of gender in the response to crystalline silica exposure, we instilled either sterile saline or freshly cracked crystalline silica into the lungs of both male and female C57BL/6 mice. There were no observable differences between saline-exposed male (Fig. 1A) and female (Fig. 1B) mice 14 days after saline instillation, whereas both male (Fig. 1C) and female (Fig. 1D) silica-exposed mice had significant pulmonary lesions. To quantify the observed histological changes, we measured total lung hydroxyproline content as a measure of collagen deposition (Fig. 1E). We observed that while silica-exposed female mice had significantly more collagen per lung than saline-exposed female mice (185.4 ± 8.8 vs. 146.7 ± 11.2), they had significantly less collagen per lung than silica-exposed male mice (185.4 ± 8.8 vs. 220.9 ± 9.1) (Fig. 1E). To ensure that the differences observed were not due to differences in lung size between genders, we performed morphometric analyses on histological sections (as described in MATERIALS AND METHODS) to measure the tissue volume density (Fig. 1F) and the percent of that tissue that is collagen (Fig. 1G). We observed that silica-exposed female mice have significantly less tissue volume density than silica-exposed male mice (0.38 ± 0.02 vs. 0.44 ± 0.02) (Fig. 1F), whereas the percentage of tissue that is collagen is not different between the sexes (4.9 ± 1.0 vs. 5.1 ± 0.7) (Fig. 1G). Importantly, although male mice are significantly larger than female mice of the same age (data not shown), we observed that the dry weights of male and female lungs were not different either after saline instillation (28.0 ± 3.0 mg vs. 28.2 ± 1.7 mg) or after silica instillation (72.3 ± 2.9 mg vs. 67.4 ± 2.2 mg).

Female mice have more inflammation in the lung than male mice in response to silica exposure. There was no difference in the number of resident inflammatory cells in the lungs of saline-exposed male and female mice (Fig. 2A) of which greater than 95% are macrophages (Fig. 2B). Both male and female mice exposed to silica had a significant increase in the numbers of inflammatory cells in the lavageable compartment of the lung (Fig. 2A). However, silica-exposed female mice had significantly more total inflammatory cells in whole lung lavage compared with males (6.8 ± 0.9 × 105 vs. 2.8 ± 1.0 × 105). There were significantly more total macrophages (Fig. 2B), neutrophils (Fig. 2C), and lymphocytes (Fig. 2D) in silica-exposed female mice compared with silica-exposed male mice. Notably, though, the percentage of macrophages (Fig. 2B), neutrophils (Fig. 2C), and lymphocytes (Fig. 2D) in the lungs of male and female silica-exposed mice was not different.

To further characterize the differences in the inflammatory response to silica observed between male and female mice, we measured the whole lung lavage content of the macrophage-specific chemotactant MCP-1, the neutrophil chemoattractant KC, and the chemokines OPG and CCL9 (also known as MIP-1γ), which are known to mediate macrophage regulation of ECM homeostasis in bone and which we have previously shown to be induced in the lung in response to both LPS and bleomycin (5). We observed that these four cytokines were undetectable in whole lung lavage consequent to saline exposure (Fig. 3, A–D). However, we observed robust increases in all four cytokines in both male and female silica-exposed mice. Notably, there was significantly more of the macrophage-specific chemokines MCP-1 (Fig. 3A) and CCL9 (Fig. 3D) in whole lung lavage fluid from female mice than from male mice, whereas there was no difference between genders in KC (Fig. 3C) or OPG (Fig. 3D).

Female mice have reduced ERα mRNA expression in lung tissue. To address the role of sex hormones in the response to silica, we first measured levels of estradiol in serum from saline- and silica-treated male and female mice. There was a trend towards increased circulating estradiol levels in saline-exposed female mice compared with saline-exposed male mice (5.2 ± 1.0 pg/ml for females vs. 3.9 ± 0.4 pg/ml for males; n = 6/group). silica had no effect on serum estradiol levels in either male or female mice (5.5 ± 1.0 pg/ml for females vs. 3.3 ± 0.6 pg/ml for males; n = 6/group). To determine whether signaling through estrogen receptors could be contributing to the observed gender differences in the response to silica instillation, we examined expression of mRNA for ERα, ERβ, and the newly described GPER gpr30 (8). We observed that while there were no differences in GPER mRNA either at baseline or in response to silica instillation (Fig. 4C) and that there were no gender-specific differences in ERβ mRNA expression (Fig. 4B), there was a significant difference in ERα mRNA expression between saline-exposed male and female mice (Fig. 4A). Additionally, silica exposure caused...
a significant decrease in ERα mRNA expression in both male and female mice (Fig. 4A).

Ovariectomy restores sensitivity of female mice to silica. To further address the role of estrogen in the response to silica, we exposed control or ovariectomized (OVX) female C57BL/6 mice to either saline or silica. We observed that silica-exposed OVX mice had more total lung hydroxyproline than silica-exposed control female mice (293.9 ± 11.1 vs. 234.7 ± 9.5; n = 6/group) (Fig. 5). Consistent with our observation, control mice had more total inflammatory cells in whole lung lavage than silica-exposed OVX mice (4.3 ± 0.4 × 10^5 vs. 2.9 ± 0.2 × 10^5) (Fig. 6A). Although there were no observable differences in the percentage of the total inflammatory cells that were either macrophages or PMNs (Fig. 6, B and C), there...
was a significant difference in total macrophage numbers between the two conditions (Fig. 6B). In addition, there were significant differences in both the percentage and total number of lymphocytes in the BALF of OVX mice compared with control (Fig. 6D). There was no difference in levels of circulating estrogen between control female and OVX female C57BL/6 mice (9.6 ± 3.3 pg/ml for control females vs. 4.4 ± 2.0 pg/ml for OVX females; n = 6/group), although again, there was a trend towards reduced circulating estrogen in the OVX females. Consistent with the observed inflammatory profiles, both silica-exposed OVX and control mice had significant increases in the cytokines MCP-1, KC, and OPG over baseline (Fig. 7, A–D). It is interesting to note that silica-exposed OVX mice had significantly less MCP-1 (Fig. 7A) in their lungs compared with control mice (459.5 ± 170.7 vs. 992.3 ± 88.2). Similarly, while there was not a significant difference in lavageable CCL9 between control and OVX mice, there was a trend towards the OVX mice having reduced total lavageable CCL9 in whole

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Fig. 2. Inflammatory cells in whole lung lavage of saline- and silica-exposed male and female C57BL/6 mouse lung. A: total inflammatory cells per mouse and number and percentage of those cells in A that are macrophages (B), neutrophils (PMNs; C), and lymphocytes (D); n = at least 5/group; *P < 0.05 vs. same gender saline-exposed, #P < 0.05 vs. silica-exposed males. Data presented are representative of 3 independent experiments.

Fig. 3. A–D: cytokines in whole lung lavage fluid from saline- and silica-exposed male and female C57BL/6 mice; n = at least 5/group; *P < 0.05 vs. same gender saline-exposed, #P < 0.05 vs. silica-exposed males. Data presented are representative of 3 independent experiments.
recent study showed that declines in lung function as measured by flexiVent consequent to bleomycin instillation are more pronounced in male C57BL/6 mice, whereas there were no observable differences between the sexes in more traditional parameters of pulmonary fibrosis including hydroxyproline and inflammation (27). A partial explanation of these sex-specific differences in bleomycin-induced fibrosis may be due to differential expression or activity of bleomycin hydrolase (27). It is currently unclear whether bleomycin hydrolase expression is modulated by estrogen. However, this raises the important question of whether other aspects of the fibroproliferative response might be modulated by estrogen.

Consistent with a previous report (13), we observed a high degree of biological variability and no statistical differences in levels of circulating estrogen between the genders or between control and OVX mice in these experiments, although a trend toward decreased estrogen levels in OVX mice was noted. A possible explanation for this observation is that levels of circulating estrogen vary by time of day (24) and during the estrous cycle (18). We did not control for progression through the estrous cycle in the current experiments that would account for these observations. However, we did observe significant differences between the genders in ERα mRNA at baseline. While measuring mRNA levels of signaling receptors is a surrogate for signaling activity itself, the differences in ERα mRNA expression at baseline between the genders suggest that signaling through the ERα may play a role in modulating the response to silica. In support of this, it has been shown that female mice clear *Paracoccidioides brasiliensis* instilled into the lung as a model of fungal infection more quickly than male mice (43). Consistent with this observation, it has been demonstrated that estrogen by itself can induce TNFα production in a macrophage cell line in vitro (15) and can increase serum concentrations of TNFα in mice (56). However, in a model of carrageenan-induced pleurisy, it was shown that addition of estrogen reduced inflammatory cell recruitment (14). Importantly, however, it was also recently shown that estrogen can modulate the inflammatory response to IL-1β in the liver but not in the lung, supporting the notion that the effects of estrogen are tissue specific (19). A more recent study has shown that, in a mouse model of allergic airways disease, female mice had significantly more inflammatory cells in whole lung lavage and concomitantly higher levels of IL-4, IL-5, and TGF-β, and the OVX females in that study had a male inflammatory response (4). These observations are fundamentally consistent with the results we show in the present study. However, in that model system, indicators of airway inflammation and fibrosis in response to crystalline silica exposure that may be dependent on expression of the ERα. Supporting this observation, we show that ovariectomy restores male-type sensitivity to silica.

Gender differences in models of lung disease in general and PF in particular have been reported previously, although with conflicting results. For example, in one study it was shown that female Fisher 344 rats are more susceptible to bleomycin-induced fibrosis than male rats (23). In that study, ovariectomized female rats developed less fibrosis than sham surgery controls, whereas estradiol rescue restored bleomycin sensitivity in the ovariectomized females (23). However, another more recent study showed virtually the opposite result in Sprague-Dawley rats (50) reminding us that genetic differences between inbred strains of laboratory animals also play a role in models of lung injury. In C57BL/6 mice, which we used in the presently reported studies, males are more sensitive than females to bleomycin-induced lung injury (27), and another recent study showed that declines in lung function as measured in lung lavage fluid (7.1 ± 0.6 vs. 4.9 ± 0.9 ng; P = 0.06) (Fig. 7D) that is consistent with the differences observed between male and female silica-exposed mice (Fig. 3D).

**DISCUSSION**

We have shown gender-specific differences in inflammation and fibrosis in response to crystalline silica exposure that may be dependent on expression of the ERα. Supporting this observation, we show that ovariectomy restores male-type sensitivity to silica.

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fibrosis including α-SM actin and collagen expression were also increased in the female mice. These observations taken together suggest that the role of estrogen in regulating the inflammatory and fibrotic response to lung injury is highly complex.

It is interesting to note that in our model system, the inflammatory response to silica exposure is inversely correlated with the fibrotic response, indicating that the severity of the inflammatory response is not a good predictor of the degree of fibrotic response in this model system. However, the results of the current study strongly support a role for estrogen in regulating important mechanisms that contribute to the development of both inflammation and fibrosis in response to crystalline silica. For example, estrogen can either promote or inhibit apoptosis (45), and, in turn, apoptosis can either promote or inhibit pathogenesis of PF depending on the cell type involved and the microenvironment of the lung (20). Thus estrogen regulation of apoptosis, and the relationship between inflammation and fibrosis, warrant further investigation.

Fig. 6. A–D: inflammatory cells in whole lung lavage of saline- and silica-exposed control or OVX female C57BL/6 mice; n = at least 5/group; *P < 0.05 vs. saline-exposed females of the same treatment, #P < 0.05 vs. silica-exposed control females. Data presented are representative of 3 independent experiments.

Fig 7. A–D: cytokines in whole lung lavage of saline- and silica-exposed control or OVX female C57BL/6 mice; n = at least 5/group; *P < 0.05 vs. saline-exposed females of the same treatment, #P < 0.05 vs. silica-exposed control females. Data presented are representative of 3 independent experiments.
The relationship between inflammation and fibrosis has been a subject of debate for many years (38). It has commonly been thought that alveolar macrophages play key roles in the pathogenesis of particle-induced lung injury (16). Interaction of silica particles with the alveolar macrophage, potentially through interaction with scavenger receptors on the macrophage cell surface (26), leads to phagocytosis and either particle clearance via the migration of the macrophage through the lymphatic system (32) or particle retention within the macrophage, resulting in the activation of the cell to produce a number of mediators (reviewed in Ref. 22) classically thought to be proinflammatory. The role of growth factors, cytokines, and other proteins typically associated with an inflammatory response have been and are still considered to be important in the overall etiology of PF (38, 44). However, the hypothesis that degree of inflammation is critically linked to the etiology of PF has now fallen out of favor. New hypotheses consider interactions between a damaged and frustrated epithelium and the response of underlying mesenchymal cells to the fibroproliferative milieu to be important (38, 44). Our current observations support the notion that the relationship between inflammation and fibrosis is complex but do not address the nature of this relationship mechanistically.

Interestingly, recent evidence from other models of fibroproliferative disease suggests that the inflammatory cells can both potentiate and resolve fibrosis. For example, in a mouse model of CCL4-induced liver fibrosis, systemic manipulation of macrophage populations can have profound effects on phenotype (17). Depleting macrophages systemically during development of liver fibrosis decreases scarring, whereas depletion of macrophages after the acute phase of injury causes enhanced fibrosis that is attributed to a failure of matrix degradation (17). These observations suggest the hypothesis that macrophages or other inflammatory cells may also participate in maintaining matrix homeostasis in the lung and may negatively regulate fibrosis.

In other tissues, ECM homeostasis is maintained by cells of myofibroblastic lineage to which macrophages belong. The best characterized of these tissues is bone, in which osteoclasts, highly specialized bone macrophages, both regulate deposition of ECM by the myofibroblast-like osteoblasts and resorption of ECM through proteolytic degradation of collagen and bone matrix (29). It was recently shown that CCL9 and the cognate receptor, CCR1, are the chemokine and receptor most abundantly produced by osteoclasts (33). CCL9 has also recently been shown to participate in osteoclast differentiation and to induce protease production in these cells (35). Additionally, CCL9 instilled into the lungs of juvenile mice induces MMP9 expression, and blocking CCL9 with an antibody attenuates hyperoxia-induced air space enlargement in newborn mouse lung (Fattman and Brass, unpublished observations). In this study, we demonstrate that both macrophage numbers (Fig. 2B) and CCL9 levels (Fig. 3D) are increased in BALF from silica-exposed female mice and that these results correspond with decreased hydroxyproline levels in these animals (Fig. 1E). Although not directly examined in this set of studies, these observations taken together are consistent with the hypothesis that the fibrotic response could be negatively regulated by components of the inflammatory response.

In summary, we have shown a gender-dependent divergent inflammatory and fibrotic response to crystalline silica that may be regulated by the effects of macrophage-specific chemokines and estrogen.

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

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GENDER INFLUENCES RESPONSE TO EXPERIMENTAL SILICA-INDUCED LUNG FIBROSIS


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