Arsenic is a toxic metalloid contaminant in drinking water sources throughout the world, affecting hundreds of millions of people (Mandal and Suzuki 2002). Exposure to arsenic via drinking water in early life has been shown to increase the risk of mortality from bronchiectasis (Smith et al. 2006). Bronchiectasis unrelated to cystic fibrosis (CF) is a chronic, progressive lung disease characterized by cough and sputum production, periodic infectious exacerbations, and premature death in adulthood (Grimmwood 2011). Bronchiectasis associated with CF is thought to arise from impaired innate immune pathways in the lung (Barker 2002), and although the pathology of non-CF bronchiectasis is poorly understood, similar mechanisms are likely to be involved (Grimwood 2011). The processes linking arsenic exposure in early life and the development of bronchiectasis are unclear.

There is evidence for an effect of \textit{in utero} arsenic exposure on innate immunity. Arsenic exposure during pregnancy induces oxidative stress and increases inflammatory cytokine levels and reduces T-cell numbers in the placenta and impairs thymic development in the infant (Ahmed et al. 2011; Raqib et al. 2009). Arsenic exposure during pregnancy has also been shown to increase the risk of lower respiratory tract infections during infancy (Rahman et al. 2011). Recurrent lower respiratory tract infections in early childhood and defective pulmonary immune defenses are important risk factors for the development of non-CF bronchiectasis (King et al. 2006). In addition, \textit{in utero} exposure to arsenic reduces birth weight, which is associated with worse respiratory health outcomes in later life (Huycck et al. 2007). Low birth weight infants are more susceptible to lower respiratory tract infections in infancy (McCall and Acheson 1968), have impaired lung function during childhood (Chan et al. 1989), and are at an increased risk of death from chronic lung diseases in adulthood (Barker et al. 1991). Taken together, these factors suggest that arsenic-induced impairments in growth and development of the lung, along with alterations in immune function, may contribute to the development of bronchiectasis in individuals exposed to arsenic \textit{in utero}.

In the present study, we investigated the effects of \textit{in utero} exposure to arsenic on postnatal lung mechanics, lung structure, and gene expression using mouse models. We hypothesized that \textit{in utero} exposure to arsenic via drinking water impairs lung development and immune pathways in the lung.

\section*{Methods}

\textbf{Animals and exposure to arsenic.} Animals were purchased from the Animal Resources Centre (Murdoch, Western Australia, Australia). Three strains of mice were used; BALB/c, C3H/HeARC, and C57BL/6. Animals were treated humanely and with regard for alleviation of suffering. All studies were conducted according to the guidelines of the National Health and Medical Research Council Australia and approved by the institutional animal ethics committee. Pregnant mice were given drinking water containing 0 (control) or 100 µg/L of arsenic from gestation day (GD) 8 until birth of their offspring (at approximately GD20) in the form of sodium arsenite (NaAsO$_2$). The arsenic concentration of drinking water was confirmed by inductively coupled plasma–mass spectrometry (ICP-MS) (Geotechnical Services, Perth, Western Australia, Australia). After their offspring were born, mothers were all given control drinking water. All mice received the same fixed formulation diet (Specialty Feeds, Glen Forrest, Western Australia, Australia). The arsenic content in the mouse chow was determined through ion chromatography ICP-MS to be 0.42 ± 0.02 µg/g total arsenic and 0.03 ± 0.001 µg/g inorganic arsenic (Centre for Environmental Risk Assessment and Remediation, University of South Australia, Mawson Lakes, South Australia, Australia). Outcomes were measured in the offspring at 2 weeks of age (BALB/c, n = 29 controls and 24 arsenic exposed; C3H/HeARC, n = 14 controls and 17 arsenic exposed; C57BL/6, n = 24 controls and 32 arsenic exposed).

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The authors declare they have no actual or potential competing financial interests.

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\section*{Environmental Health Perspectives}

\textbf{Bronchiectasis unrelated to cystic fibrosis (CF) is a chronic, progressive lung disease characterized by cough and sputum production, periodic infectious exacerbations, and premature death in adulthood (Grimmwood 2011). Bronchiectasis associated with CF is thought to arise from impaired innate immune pathways in the lung (Barker 2002), and although the pathology of non-CF bronchiectasis is poorly understood, similar mechanisms are likely to be involved (Grimwood 2011). The processes linking arsenic exposure in early life and the development of bronchiectasis are unclear.}

\begin{itemize}
  \item Exposure to arsenic via drinking water is a global environmental health problem.
  \item In \textit{utero} exposure to arsenic via drinking water increases the risk of lower respiratory tract infections during infancy and mortality from bronchiectasis in early adulthood.
  \item OBJECTIVES: We aimed to investigate how arsenic exposure in early life alters lung development and pathways involved in innate immunity.
  \item METHODS: Pregnant BALB/c, C57BL/6, and C3H/HeARC mice were exposed to 0 (control) or 100 µg/L arsenic via drinking water from gestation day 8 until the birth of their offspring. We measured somatic growth, lung volume, and lung mechanics of mice at 2 weeks of age. We used fixed lungs for structural analysis and collected lung tissue for gene expression analysis by microarray.
  \item RESULTS: The response to arsenic was genetically determined, and C57BL/6 mice were the most susceptible. Arsenic-exposed C57BL/6 mice were smaller in size, had smaller lungs, and had impaired lung mechanics compared with controls. Exposure to arsenic \textit{in utero} up-regulated the expression of genes in the lung involved in mucus production (Cgla3, Muc5b, Scgb3a1), innate immunity (Reg3γ, Tlf2, Dynlr2, Lplunc1), and lung morphogenesis (Sox2). Arsenic exposure also induced mucus cell metaplasia and increased expression of CLCA3 protein in the large airways.
  \item CONCLUSIONS: Alterations in somatic growth, lung development, and the expression of genes involved in mucociliary clearance and innate immunity in the lung are potential mechanisms through which early life arsenic exposure impacts respiratory health.
  \item KEY WORDS: gene expression, growth and development, innate immunity, mucociliary clearance, toxicity. Environ Health Perspect 121:244–250 (2013). dx.doi.org/10.1289/ehp.1205590 [Online 4 December 2012]
\end{itemize}
**Thoracic gas volume (TGV) and lung mechanics.** To measure lung mechanics in vivo, mice were anesthetized, tracheotomized, and mechanically ventilated. Plethysmography was used to measure TGV as described previously (Janosi et al. 2006). Lung mechanics were measured using the forced-oscillation technique as described previously (Sly et al. 2003). [For further details, see Supplemental Material, p. 2 (http://dx.doi.org/10.1289/ehp.1205590).]

**Stereological analysis of lung structure.** Lung structure was assessed using stereology techniques according to ATS/ERS (American Thoracic Society/European Respiratory Society) guidelines (Hsia et al. 2010). [For further details, see Supplemental Material, p. 3 (http://dx.doi.org/10.1289/ehp.1205590).]

**Gene expression profiling studies.** Peripheral lung tissue was excised from mice and stabilized in RNA Later (Qiagen, Hilden, Germany) (n = 6 litters per treatment group, per strain). The lung tissue was disrupted in TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) employing a rotor stator homogenizer and extracted with TRIzol followed by RNA purification using RNeasy (Qiagen). The quality of the RNA samples was assessed on the bioanalyzer (model 2100; Agilent Technologies, Santa Clara, CA, USA) [RNA integrity number (RIN) 8.30 ± 0.58]. Total RNA samples were labelled and hybridized to Mouse Gene STI.10 microarrays (Affymetrix, Santa Clara, CA, USA) by the Molecular Genetic Research Services, at the Australian Neuromuscular Research Institute, QEI Medical Centre, Nedlands, Western Australia, Australia.

The raw microarray data were preprocessed employing the PLIER+16 algorithm (gcbg background subtraction, quantile normalization, iterPLIER summarization) in Expression Consol software (Affymetrix) (Bosco et al. 2009). Two low-quality microarrays were removed from the analysis. To identify differentially expressed genes, the data were analysed with moderated t-statistics (LIMMA R package; http://www. R-project. org) (Smyth 2004) and plotted as Volcano plots [see Supplemental Material, Figure S1 (http://dx.doi.org/10.1289/ehp.1205590)].

Genes with an absolute moderated t-statistic of > 3.5 and fold change > 2 were considered differentially expressed. The microarray data are available from the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE37831. A subset of differentially expressed genes was selected using the forced-oscillation technique as described previously (Sly et al. 2003). [For further details, see Supplemental Material, Table S1 (http://dx.doi.org/10.1289/ ehp.1205590).]

**Results**

**Maternal and birth outcomes.** There was no difference in water consumption between mothers given 100 µg/L arsenic compared with control mothers for any strain of mouse (BALB/c, p = 0.46; C3H/HeARC, p = 0.34; C57BL/6, p = 0.82) [see Supplemental Material, Table S1 (http://dx.doi.org/10.1289/ ehp.1205590)]. There were no differences in litter size (BALB/c, p = 0.30; C3H/HeARC, p = 0.87; C57BL/6, p = 0.54) or gestational age at birth (BALB/c, p = 0.84; C3H/HeARC, p = 0.21; C57BL/6, p = 0.31) between arsenic-exposed and control mice. Arsenic-exposed C57BL/6 offspring were smaller in birth weight and birth length compared with control C57BL/6 offspring [p < 0.001, respectively (see Supplemental Material, Table S1)]. There were no differences in birth weight or length between arsenic-exposed mice and control BALB/c (p = 0.15 and p = 0.21) or C3H/HeARC offspring (p = 0.18 and p = 0.78). For all analyses reported there was no difference in responses between male and female offspring, so data were pooled.

**TGV and lung mechanics.** Arsenic-exposed C57BL/6 mice were significantly smaller in body weight (p < 0.001) than control offspring at 2 weeks of age and had significantly lower TGV than controls (p < 0.001), even after adjusting for snout-vent length (p = 0.04) [see Supplemental Material, Figure S2 (http://dx.doi.org/10.1289/ ehp.1205590)]. Arsenic-exposed C57BL/6 mice had significantly higher tissue damping and tissue elastance (p < 0.001 in both cases) compared with C57BL/6 controls (Figure 1). These differences were maintained after adjusting for TGV (p = 0.006 and p = 0.004, respectively). There was no effect of arsenic on airway resistance (p = 0.29) in C57BL/6 mice, which was maintained after adjusting for TGV (p = 0.78). In C3H/HeARC mice there was no difference in body weight (p = 0.38) at 2 weeks of age for arsenic-exposed mice compared with control mice. TGV was significantly higher in arsenic-exposed mice compared with controls (p = 0.02), which was maintained after adjusting for snout-vent length (p < 0.001) [see Supplemental Material, Figure S2 (http://dx.doi.org/10.1289/ ehp.1205590)]. At 2 weeks of age, arsenic-exposed C3H/HeARC mice had significantly higher airway resistance compared with controls (p = 0.04), which was still higher after adjusting for TGV (p = 0.002). There was no effect of arsenic on tissue damping or tissue elastance (p = 0.46 and p = 0.34, respectively) even after adjusting for TGV (p = 0.15 and p = 0.65, respectively) (Figure 1).

In BALB/c mice there was no difference in body weight (p = 0.31) for arsenic-exposed mice compared with control mice at 2 weeks of age. TGV was significantly higher in mice exposed to arsenic in utero (p = 0.05); however, there was no difference after adjusting for snout-vent length (p = 0.20) [see Supplemental Material, Figure S2 (http://dx.doi.org/10.1289/ ehp.1205590)]. In BALB/c mice there was no difference in airway resistance at 2 weeks of age between the groups (p = 0.82), which was also the case after adjusting for TGV (p = 0.28). There was evidence to suggest that both tissue damping and tissue elastance were lower in arsenic-exposed mice compared with controls (p = 0.07 and p = 0.02, respectively); however, these differences were negated after adjusting for TGV (p = 0.65 and p = 0.22, respectively) (Figure 1).

**Stereological analysis of lung structure.** In C57BL/6 mice, lung volume (calculated by stereology) was significantly smaller in
arsenic-exposed offspring compared with controls (p = 0.03). The number of alveoli in the lung (p = 0.01) and the surface area of the lung (p = 0.04) were also smaller in arsenic-exposed C57BL/6 mice compared with control mice of the same strain; however, there was no difference in the volume of the alveoli (p = 0.41) (Figure 2). We found no differences in any of the structural parameters measured between arsenic-exposed mice and control BALB/c mice or C3H/HeARC.

**Gene expression profiling studies.** Using microarray analysis, we identified 10 annotated genes in the lung that were differentially expressed as a result of in utero exposure to arsenic (Table 1). C57BL/6 mice had 7 genes that were differentially expressed, whereas C3H/HeARC mice had 2 and BALB/c mice had only 1 differentially expressed gene(s). Only 1 gene was down-regulated in response to arsenic, Olfr1274 (olfactory receptor 1274), in the C57BL/6 strain. All of the other genes identified as differentially expressed were up-regulated in response to arsenic exposure.

Three of the 10 genes that were up-regulated ([Clca3, Muc5b, and Sgbh3a1](secretoglobin, family 3A, member 1)) were involved in the regulation and/or secretion of mucus in the airways (Roy et al. 2011). These genes were all up-regulated in C57BL/6 mice. Four of the 10 genes [Lplunc1 (long palate, lung, and nasal epithelium carcinoma-associated protein 1), Tff2 (trefoil factor 2 (spasmolytic protein 1)), Reg3γ, and Dynlrb2 (dynemin light chain roadblock-type 2)] that were up-regulated are involved in innate immune function (Bingle and Craven 2002; Jin et al. 2009; McAleer et al. 2011; Nikolaidis et al. 2003). Lplunc1, Tff2, and Reg3γ were up-regulated in C57BL/6 mice, and Dynlrb2 was up-regulated in C3H/HeARC mice. We also identified a gene involved in lung branching morphogenesis and epithelial cell differentiation, Sox2 (Gontan et al. 2008), which was up-regulated in C3H/HeARC mice. In BALB/c mice, an oncogene [Sedb10 (synovial sarcoma, X member B, breakpoint 10)] was the only gene up-regulated (Chen et al. 2003).

**Validation of array expression using qRT-PCR.** Using qRT-PCR, we validated the expression of four genes in C57BL/6 mice ([Clca3, Muc5b, Tff2, and Reg3γ] and one gene in C3H/HeARC mice [Sox2 (SRY-box containing gene 2)]). In all cases, the expression patterns, normalized to the Eef1a1 gene, correlated to those obtained in the microarray analysis (Figure 3).

**Quantification of mucus and protein in the airways.** We used Alcian blue–periodic acid–Schiff staining and immunohistochemistry to quantify mucus-producing cells and CLCA3, MUC5B, and REG3γ protein in the airways of BALB/c, C3H/HeARC, and C57BL/6 mice (Figure 4). We found that arsenic exposure in utero caused mucous cell metaplasia in the large (p < 0.001) and medium (p = 0.02) airways of C57BL/6 mice and increased the expression of CLCA3 (p < 0.01) and REG3γ (p = 0.02) proteins in the large airways of C57BL/6 mice (Figure 5). The levels of MUC5B protein were low and not differentially expressed between the airways of...
arsenic-exposed mice and control C57BL/6 mice. There were no differences in the number of mucus-producing cells or CLCA3, MUC5B, or REG3γ proteins between the airways of arsenic and control mice of either the BALB/c or C3H/HeARC strains (data not shown).

**Discussion**

Exposure to arsenic in utero has been shown to increase morbidity from lower respiratory tract infections in infancy and increase mortality from bronchiectasis in adulthood (Rahman et al. 2011; Smith et al. 2006). In the present study, our results show that the response to arsenic in mice is genetically determined and that the C57BL/6 strain is the most susceptible of the three strains studied. In utero exposure to arsenic impaired somatic growth, lung volume, and parenchymal lung mechanics in C57BL/6 mice. In addition, exposure to arsenic during gestation is capable of altering the expression of genes that function in mucociliary clearance, innate immunity, and lung growth in the offspring. Arsenic also induced mucous cell metaplasia and increased expression of CLCA3 protein in the airways of C57BL/6 mice. Increased mucus secretion and altered lung growth may impair the ability of the airways to clear pathogens and thus provide a potential mechanism by which exposure to arsenic increases the risk of developing chronic localized infections leading to bronchiectasis.

Exposure to arsenic in utero impaired intratracheal growth and lung development. Arsenic-exposed C57BL/6 mice were small for gestational age; had lower lung volume, lung surface area, and alveolar number; and had impaired lung mechanics compared with control mice of the same strain. This finding supports results from a recent study that also found that C57BL/6 mice exposed to arsenic in utero had deficits in growth in early life (Kozul-Horvath et al. 2012). Impairments to body growth and development were observed in the offspring of arsenic-exposed mothers. Exposure to arsenic in utero impaired lung mechanics in the offspring of arsenic-exposed mothers. This finding is consistent with previous studies that have demonstrated the impact of in utero exposure to arsenic on lung development.

**Table 1. Genes altered by arsenic exposure.**

<table>
<thead>
<tr>
<th>Mouse strain/ gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Reference</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Cica3</td>
<td>Chloride channel calcium activated 3</td>
<td>Regulation of mucus production and secretion</td>
<td>Zhou et al. 2002</td>
</tr>
<tr>
<td></td>
<td>Lplunc1</td>
<td>Long palate, lung, and nasal epithelium carcinoma-associated protein 1</td>
<td>Innate immunity in mouth, nose, and lungs</td>
<td>Bingle and Craven 2002</td>
</tr>
<tr>
<td></td>
<td>Reg3γ</td>
<td>Regenerating islet-derived 3 gamma</td>
<td>Innate immunity–antibacterial properties in the mucus secretions</td>
<td>McAlear et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Scgb3a1</td>
<td>Secretoglobin, family 3A, member 1</td>
<td>Secretory cell subtype in the lung</td>
<td>Roy et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Tff2</td>
<td>Trefoil factor 2 (spasmolytic protein 1)</td>
<td>Innate immunity–allergen induced gene, expressed in mucous cells</td>
<td>Nikolaidis et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Muc5b</td>
<td>Mucin 5, subtype B, tracheobronchial</td>
<td>Major mucin in respiratory mucus</td>
<td>Kirkham et al. 2002</td>
</tr>
<tr>
<td></td>
<td>Olf1274</td>
<td>Olfactory receptor 1274</td>
<td>Offaction</td>
<td>Zhang and Firestein 2002</td>
</tr>
<tr>
<td></td>
<td>C3H/HeARC</td>
<td>Synovial sarcoma, X member B, breakpoint 10</td>
<td>Lung branching morphogenesis and epithelial cell differentiation</td>
<td>Cancer</td>
</tr>
</tbody>
</table>

**Table 2. Genes altered by arsenic exposure.**

<table>
<thead>
<tr>
<th>Mouse strain/ gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Reference</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Muc5b</td>
<td>Synovial sarcoma, X member B, breakpoint 10</td>
<td>Lung branching morphogenesis and epithelial cell differentiation</td>
<td>Cancer</td>
</tr>
</tbody>
</table>
size and lung size during infancy may result in airways and alveoli that are prone to closure and collapse, thus increasing the susceptibility to obstruction and increased pathogen load. Low birth weight is associated with impaired lung function and increased mortality from obstructive lung disease (Barker et al. 1991; Hulskamp et al. 2009). A potential mechanism for the observed intrauterine growth restriction may be oxidative stress caused by arsenic-induced production of reactive oxygen species inducing placental insufficiency (Vahter 2007). This proposed mechanism is supported by the knowledge that the mouse strain most sensitive to arsenic, C57BL/6, is highly susceptible to the oxidative stress effects of cigarette smoke and bleomycin compared with other strains (Chen et al. 2001a; Yao et al. 2008). There is evidence from cigarette smoke exposure models that intrauterine growth restriction alone can cause alterations in early life lung function (Larcombe et al. 2011). It is therefore possible that the effects of arsenic exposure on body weight, lung function, and lung size in the present study, in the C57BL/6 mice at least, were due to arsenic altering the nutritional status of the offspring. However, we also identified responses, such as alterations in lung gene expression and airway morphology that seem to be unique to arsenic exposure and have yet to be linked with altered nutritional status. It is clear that exposure to arsenic during pregnancy is having a significant effect on intrauterine growth, which may predispose infants to chronic lung diseases through impairments in lung growth and development.

We found that arsenic exposure during pregnancy was capable of altering the expression of genes in the lung at 2 weeks of age. The differentially expressed genes identified are involved in mucus production, innate immunity, and lung morphogenesis pathways. The gene that was most highly up-regulated was the calcium-activated chloride channel, Clca3 gene. This gene has been detected in goblet cells within the tracheal and bronchial epithelium of mice when metaplasia of mucous cells is present (Leverkoehne and Gruber 2002) and is thought to play a role in the mucus secretion seen in chronic obstructive pulmonary disease (COPD) and CF in humans (Rogers 2003; Shale and Ionescu 2004). Blocking the production and/or activity of Clca3 can inhibit the production of mucus, whereas up-regulation can enhance mucus production (Zhou et al. 2002). Clca3 has been implicated in the regulation of mucus secretion by controlling the packaging and/or release of secreted mucins such as MUC5AC and MUC5B, which are the major gel-forming mucins in respiratory secretions (Nakanishi et al. 2001; Zhou et al. 2002). MUC5B levels are increased in sputum from the airways of patients with COPD and CF compared with
arsenic exposure impairs lung development

In utero arsenic exposure impairs lung development

Conclusions

In the present study, we have shown that in utero exposure to arsenic via drinking water impairs lung development, resulting in reduced lung size and impaired lung structure and function. The response to arsenic is genetically determined and the C57BL/6 strain is the most susceptible. Arsenic exposure in utero altered the expression of genes that regulate innate immunity, mucus production, and morphogenesis of the lung. Arsenic-induced alterations in the development of lung structure and innate immunity may reduce the ability to clear respiratory pathogens during a period of high susceptibility in infancy (Simoes 1999). Because respiratory infections are one of the leading causes of mortality in children under 5 years of age in developing countries (WHO 2011b), arsenic-induced increases in susceptibility to infection can have devastating effects on infant morbidity and mortality in affected areas. In addition, recurrent respiratory infections throughout life can contribute to the development of chronic lung diseases, such as bronchiectasis, in adulthood. The evidence from our mouse model is that in utero exposure to arsenic impairs lung development, resulting in altered structure, function, and gene expression in infancy. Further investigation into how arsenic affects lung development in humans will be essential to understand and prevent escalating morbidity and mortality from respiratory disease in arsenic-exposed populations.

REFERENCES


