The role of cardiovascular disease-associated iron overload in Libby amphibole-induced acute pulmonary injury and inflammation

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Abstract
Pulmonary toxicity induced by asbestos is thought to be mediated through redox-cycling of fiber-bound and bioavailable iron (Fe). We hypothesized that Libby amphibole (LA)-induced acute lung injury will be exacerbated in rat models of cardiovascular disease (CVD)-associated Fe-overload and oxidative stress. Healthy male Wistar Kyoto (WKY), spontaneously hypertensive (SH) and SH heart failure (SHHF) rats were intratracheally instilled with 0.0, 0.25 or 1.0 mg/rat LA and examined at 1 day, 1 week or 1 month. Although histologically it was not possible to distinguish severity differences between strains in LA-induced initial inflammation and later fibrosis, quantitative assessment of biomarkers showed strain-related differences. LA-induced neutrophilic inflammation was reversible in WKY but persisted more in SH and SHHF. Lung MIP-2 mRNA increased only in WKY at 1 day in response to LA but not in SH and SHHF. Bronchoalveolar lavage fluid (BALF) protein increased in SH but not WKY at 1 week and 1 month, while γ-glutamyltransferase and N-acetyl-β-D-glucosaminidase activities increased in all strains (WKY>SH=SHHF). BALF ferritin levels were high at baseline and increased following LA exposure only in SH and SHHF. Ferritin heavy chain mRNA increased only in SHHF at 1 day. At 1 month ferritin light chain mRNA declined from already high baseline levels in SHHF but increased in WKY and SH suggesting its differential involvement in LA-induced injury in Fe-overload. Unlike WKY, both SHHF and SH failed to increase the lung lining antioxidant, ascorbate, in response to LA. We conclude that underlying CVD-associated Fe-overload is likely linked to persistent lung injury, inflammation and antioxidant decompensation following LA exposure in rats.

Keywords: Libby Amphibole, Iron, Inflammation

Introduction
Asbestos exposure in general is known to cause pulmonary toxicity leading to a variety of diseases including asbestosis, mesothelioma and lung cancer. Miners and residents in the town of Libby, Montana, have increased incidences of asbestos-related diseases, such as asbestosis and mesothelioma, associated with occupational and/or environmental exposure to amphibole contaminated vermiculite during mining (Peipins et al., 2003; Sullivan, 2007). The Libby amphiboles (LA) are a
mixture of winchite, richterite, tremolite and other minerals in prismatic, acicular and asbestiform morphologies (Meeker et al., 2003). Because physicochemically diverse fibers can induce various diseases with varying potencies, toxicological studies have begun focusing on how the LA fiber mix might cause toxicity differently than fibers that have been studied for decades (Putnam et al., 2008; Duncan et al., 2010).

Toxicity due to inhalation of asbestos fibers has been linked to the production of reactive oxygen species (ROS) both directly by the fibers and indirectly by activation of inflammatory cells (Sanchez et al., 2009). ROS cause cellular damage through oxidation of lipids, proteins and DNA. This damage leads to activation of cell signaling pathways and transcription factors that can cause a variety of alterations in gene expression related to antioxidants, apoptosis, cytokines, growth factors and stress response (Kamp and Weitzman, 1999). The inability to clear fibers, such as amphiboles, resulting in their biopersistence, causes the lung to exist in a state of chronic oxidative stress and inflammation due to the prolonged activation of inflammatory cells and the generation of ROS.

Fe has a considerable role in the production of ROS by asbestos fibers. It has been postulated that Fe associated with the surface of fibers has the ability to support Fenton reactions using reducing equivalents, such as airway lining ascorbate, thereby generating ROS (Ghio et al., 2008). Furthermore, modulation of the amount of Fe on the surface of the fiber alters ROS production and consequently the toxicity of fibers (Gazzano et al., 2007a). Chelation of Fe reduces the generation of ROS upon exposure to asbestos in vitro (Kamp et al., 1995a) and in vivo (Kamp et al., 1995b). Asbestos binds endogenous chelatable Fe in vivo upon deposition into the airways (Ghio et al., 2004). Once associated with the fiber, this additional Fe from the host may augment toxicity by potentiating ROS production (Ghio et al., 2006). Thus, it is likely that bioavailable host Fe can modify the toxicity of asbestos fibers.

Under oxidative stress, cellular Fe is generated by heme oxygenase-1 (HO-1) catalyzed degradation of heme (Raval and Lee, 2010). This process is known to transcriptionally activate production of ferritin, an intracellular Fe-binding protein which is highly critical in regulating cellular oxidative stress and a number of cellular processes catalyzed by Fe, such as the activity of mitochondrial aconitase (Gozzelino et al., 2010). Systemically Fe homeostasis is maintained by a highly abundant extracellular Fe transport protein, transferrin (Kibel et al., 2008). A number of cell membrane Fe and metal transporters are involved in tightly regulating levels of chelatable nonheme Fe (Anderson and Vulpe, 2009). In many chronic diseases the regulation of Fe homeostasis is disrupted, especially in cardiovascular (CVD), where hemoglobin is vulnerable to degradation by constant shear stress on peripheral vessels (Yasuda et al., 2002). In chronic conditions, individuals with CVD exhibit systemic tissue Fe-overload, including the lung (Kruszewski, 2004; Xie et al., 2008; Ellervik et al., 2010). Underlying oxidative stress and a dysregulation of Fe homeostasis lead to increased systemic nonheme Fe levels (Kruszewski, 2004). Due to the essential role of Fe in catalytic activity of many proteins, it is possible that the disruption of Fe homeostasis by its binding to asbestos fibers over time increases the pulmonary toxicity more readily in those having Fe-overload.

We have recently characterized dysregulation of pulmonary Fe homeostasis in two rat models of human CVD: the spontaneously hypertensive (SH) and the spontaneously hypertensive heart failure (SHHF) (Shannahan et al., 2010). As with human CVD, these rat models (SHHF > SH) have greater transcriptional activation of lung ferritin at mRNA and protein level together with increased HO-1 relative to healthy rats. Also associated with these complications, SH and SHHF exhibit an increase in chronic inflammation, lower antioxidant levels and oxidative stress in their lungs at baseline when compared to healthy Wistar Kyoto (WKY) rats (Shannahan et al., 2010). Attributable to the ability of asbestos to complex host-tissue Fe, we believe that SH and SHHF, with inherent Fe-overload, are appropriate for investigating the role of Fe in pulmonary injury caused by fibers. We hypothesized that SH and SHHF rats will exhibit greater and prolonged lung injury and inflammation upon exposure to LA, relative to healthy WKY rats, due to their pre-existent dysregulation of Fe homeostasis. To address this hypothesis, we exposed WKY, SH, SHHF to LA and examined changes in biomarkers associated with Fe homeostasis, inflammation and oxidative stress.

Materials and methods
Libby amphibole
The LA sample was collected from the Rainy Creek Complex near Libby, Montana, in 2007 by the United States Geological Survey and was processed to produce inhalable material by Meeker et al. (2003). The sample was further size fractionated by water elutriation as described previously (Webber et al., 2008) in order to isolate a rat respirable fraction (PM2.5) using a settling velocity of 3.4×10⁻⁴ cm s⁻¹. Transmission electron microscopy showed that 97.8% (135/138) of elutriated fibers had aspect ratios ≥5 consistent with the composition of LA obtained from the 2000 sample (Meeker et al., 2003;Webber et al., 2008) and with the composition of LA obtained from the PM2.5 elutriated fraction of this sample (Lowers and Bern, 2009). Fiber dimensions, as determined using transmission electron microscopy of the elutriated LA 2007 sample, were mean length 4.99±4.53 µm and width 0.28±0.19 µm; median length = 3.59 µm, width = 0.23 µm with upper and lower values of length being 0.52–27.30 µm and width 0.07–1.15 µm. The estimated fiber count for 1 mg LA sample was 218×10³. In comparison to the elutriated LA 2007 sample used in this study, air samples from Libby, Montana, have been shown to contain fibers having a mean length of 7.64±8.40 µm.
and width 0.51 ± 0.46 µm, and median length 5.2 µm and width 0.39 µm with upper and lower values of length being 0.5–195 µm and width 0.01–10 µm; with an aspect ratio of ≥5 [United States Environmental Protection Agency (US EPA), 2010]. The median fiber length being smaller than the mean length suggests that there are likely a larger proportion of fibers that are smaller than the mean length.

Animals

Male, 11–12-week old, healthy WKY, SH and obese SHHF rats were purchased from Charles River Laboratories, Raleigh, NC. All rats were maintained in an isolated room in an Association for Assessment and Accreditation of Laboratory Animal Care approved animal facility at 21 ± 1°C, 50 ± 5% relative humidity and 12 h light/dark cycle. Rats were housed (two/cage) in polycarbonate cages containing β-chip bedding. Animals received standard (5001) Purina rat chow (Brentwood, MO) and water ad libitum. The US EPA NHEERL Institutional Animal Care and Use Committee approved the protocol.

Intratracheal instillation of LA

Since the rat respirable sample of LA was prepared using the water elutriation method, a uniform saline suspension of LA for intratracheal instillation was achieved by vortexing followed by water bath sonication for 15 min. The suspension was vortexed prior to each instillation to ensure that fibers did not settle at the bottom of the tube. In order to ensure the fibers did not interact with any biological molecules or chemical dispersants of the medium prior to landing on the airway surface, we decided not to use a biological dispersion media or any other chemicals for preparing the instillate. Rats (WKY n = 12/time point; SH n = 6/time point; SHHF n = 6/time point) were anesthetized with isoflurane and intratracheally instilled with 300 µl saline containing either 0, 0.25 or 1.0 mg of LA as described previously (Wallenborn et al., 2009). The concentrations selected although high were, in general, consistent with instillation studies using other fiber types (Hirano et al., 1988; Adamson and Bakowska, 2001). Doses were chosen to assure a response in the lung upon instillation allowing for a comparative analysis between strains. Theoretically, a rat will deposit 0.07 mg of fibers during 6 h inhalation at 10 mg/m3 based on the assumption that minute volume is 200 ml and the deposition fraction to pulmonary region is 0.10. Intratracheal instillation ensured the delivery of exact concentrations of LA into the lung and allowed us to control for likely strain-related deposition differences due to their variation in breathing parameters (Shannahan et al., 2010). In our previous study, ~30% of WKY presented with nonpathogenic cardiac hypertrophy (Shannahan et al., 2010). Therefore WKY group size was increased (n = 12) to eliminate the data from those with hypertrophic hearts (heart weight >1.3 g; normal heart weight ~1 g) and still maintain appropriate group sizes for statistical comparisons.

Necropsy, sample collection and analysis

Rats were weighed [WKY 280 ± 3.46 g, SH 283 ± 3.43 g and SHHF 432 ± 4.94 g (values mean weight ± SE)] and anesthetized with an overdose of sodium pentobarbital (Virbac AH, Inc., Fort Worth, TX; 50–100 mg/kg, ip) 1 day, 1 week or 1 month following instillation of LA. Blood was collected though the abdominal aorta into blood collection tubes, which were used for a different study. Blood was then removed from the vasculature of the lung by perfusion of Ca2+/Mg2+ free phosphate buffered saline (PBS) via the pulmonary artery to avoid interference in data analysis involving Fe-binding proteins and capacities.

The trachea was then cannulated and the left lung lobe tied off. The right lung lobes were lavaged with Ca2+/Mg2+ free PBS (pH 7.4, at 37°C) equal to 35 ml/kg body weight (representing total lung capacity) × 0.6 (right lung capacity being 60% of total lung capacity). The lavage volume was kept the same for SHHF as the mean value for WKY rats on the assumption. The lung lobes were lavaged three times with the same PBS aliquot. The right lung lobes were then removed, placed in liquid nitrogen and stored at −80°C for later analysis. The left lung was fixed at 40% of its total capacity by instillation of 10% neutral buffered formalin through the trachea and stored in 10% formalin at 4°C until processed for histopathology.

Cell differential and BALF analysis

Aliquots of bronchoalveolar lavage fluid (BALF) were taken for total cell counts (Coulter Inc., Miami, FL), cell differentials and analyses of lung injury markers. Cell differentials were conducted by Cytospin preparation (Shandon, Pittsburgh, PA), and slides were stained with LeukoStat (Fisher Scientific Co., Pittsburgh, PA). Macrophages and neutrophils were counted under light microscopy and quantified based on total cell count. The remaining cell-free BALF was evaluated for the following: total protein (Coomassie plus Protein Assay Kit; Pierce, Rockford, IL), albumin (DiaSorin, Stillwater, MN), lactate dehydrogenase (LDH) activity (Thermo Fischer Diagnostics, Middletown, VA), N-acetyl-β-D-glucosaminidase (NAG) activity (Roche Diagnostics, Indianapolis, IN), γ-glutamyl transferase (GGT) activity (Thermo Fischer Diagnostics), and the Fe-binding proteins, ferritin (Kamiya Biomedical Company, Seattle, WA) and transferrin (Trf) (DiaSorin, Stillwater, MN). Total Fe-binding capacity (TIBC) was calculated by adding the unsaturated Fe-binding capacity (UIBC) (UIBC Assay; Genzyme Diagnostics, Charlottetown, Prince Edward Island, Canada) and the nonheme Fe (Serum Fe-SL Assay; Genzyme Diagnostics, Charlottetown, Prince Edward Island, Canada). All assays were adapted for BALF analysis and ran using the Konelab Arena 30 clinical analyzer (Thermo Chemical Lab Systems, Espoo, Finland). Data were normalized to volume of BALF.

One aliquot of BALF was mixed with an equal volume of 6% perchloric acid and ascorbate was measured by HPLC (C-18 Bondpack column; Millipore Waters...
Chromatography, Milford, MA) using amperometric electrochemical detection (Bioanalytical Systems, West Lafayette, IN). Total glutathione levels were measured using 5,5,9-dithiobis (2-nitro-benzoic) acid-glutathione disulfide reductase recycling assay via the Konelab clinical analyzer (Anderson, 1985).

**Lung tissue analysis**

Ascorbate and glutathione levels were measured in the caudal lung lobes. Individual samples were homogenized in 3% perchloric acid, and supernatants were analyzed for ascorbate and glutathione as described above. Caudal lung lobes were homogenized in Tris-HCl buffer (pH 7.4) with protease inhibitors present. The individual samples were then centrifuged (14,000g) for 20 min (4°C) and the supernatant stored at −80°C. The supernatant was used to determine total protein content, ferritin, Trf, nonheme Fe, TIBC and UIBC as stated above. Data were normalized to total protein content of tissues.

**RNA isolation and real-time quantitative PCR**

Total RNA was isolated from caudal lung lobes frozen in liquid nitrogen using RNaseasy mini kit (Qiagen, Valencia, CA). One-step real-time polymerase chain reaction (RT-PCR) was carried out using Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions (Shannahan et al., 2010). PCR was performed for 18S ribosomal RNA (control), macrophage inflammatory protein (MIP-2), heme-oxygenase-1 (HO-1), transferrin (Trf), ferritin light chain (FLC), ferritin heavy chain (FHC) and divalent metal transporter-1 (DMT-1). Primers for these mRNA were purchased from Applied Biosystems, Inc. (Foster City, CA). Relative fold mRNA expression was calculated for all groups considering the saline exposed WKY values as control.

**Lung histopathology**

The right lung lobe was trimmed, embedded in paraffin, sectioned to a thickness of ~3 µm (transverse), and stained with hematoxylin and eosin (H&E). The lung lesions were evaluated with particular attention to the lesion location (i.e., bronchi, terminal bronchi, alveolar duct, alveoli, interstitium, centriacinar regions, pleura). The pathological evaluation took into consideration the characteristics of each inflammatory cellular component, that is, polymorphonuclear cells, macrophages, fibrosis, microgranulomas, as well as changes in the alveolar epithelium. Histopathological changes were scored using semiquantitative grading at five levels (0 = normal; 1 = minimal; 2 = mild; 3 = moderate; 4 = severe) taking into consideration the degree of severity and the type of lesion (Shackelford et al., 2002; Nyska et al., 2005).

**Statistical analysis**

Data are expressed as mean ± SE (WKY, n=8–12/treatment group; SH, n=6/treatment group; SHHF, n=6/treatment group). Sigma Stat version 3.5 (Systat Software, Inc., Point Richmond, C.A.) was used to determine statistical comparisons via a two-way analysis of variance with strain and exposure as factors followed by a post-hoc comparison using the Holm–Sidak method. Statistical significance was determined when p was found to be less than or equal to 0.05 between treatment groups and strains.

**Results**

**Pulmonary inflammation and injury**

In order to quantify acute inflammation and injury response following LA exposure and to determine the baseline strain differences, BALF samples were analyzed for various biomarkers. Baseline levels (saline control) of BALF protein were significantly higher in SH and SHHF when compared to WKY (SHHF > SH > WKY) (Figure 1), consistent with our previous study (Shannahan et al., 2010). Surprisingly, at 1-day post-exposure no increases in BALF protein occurred in WKY or SH while SHHF rats exposed to LA demonstrated marked variability and decreases compared to strain matched control. By 1 week, only SH rats (with moderate baseline increase in protein relative to WKY) exposed to LA showed concentration-dependent increases in total protein, which persisted to 1 month. Because of the already very high levels at baseline, the LA-induced changes in BALF protein for 1 week and 1 month were not distinguishable in SHHF rats. Albumin levels were consistent with the trends demonstrated by total protein at all time points. GGT activity was significantly increased in a concentration-dependent manner and regardless of protein levels in BALF in all strains at 1 day; however, the changes in LA-exposed WKY were significantly greater when compared to LA-exposed SH and SHHF rats at 1 day and 1 week (Figure 1). At 1 week through 1 month, GGT activity still remained significantly elevated after 1 mg LA exposure in all strains, but the magnitude of effect decreased in a time-dependent manner. Concentration-dependent increases in LDH activity were observed at the same level in all strains at 1 day (Figure 1). At 1 week and continuing to 1 month, LDH activity remained elevated in all strains; however, the increase was more pronounced in SHHF when compared WKY. NAG activity, a marker of phagocyte activation increased in a concentration-dependent manner after LA exposure in all three strains at 1 day and 1 week with no remarkable strain differences and had returned to baseline by 1 month (Figure 1). Despite markedly higher baseline BALF protein in SH and SHHF, the baseline levels of these enzyme markers in most instances were comparable between strains.

All strains exhibited robust neutrophilic influx in response to LA exposure at 1 day (Figure 2). SHHF exposed to 1 mg LA had a slightly greater number of neutrophils in their BALF compared to WKY receiving the same dose (SHHF > SH > WKY). Although to a lesser
extent than the 1-day time point, neutrophil increases persisted in SH and SHHF rats at 1 week and 1 month (SH > SHHF). However, in the case of WKY, the initial robust increase in neutrophils at 1 day was largely reversed by 1 week. In WKY, numbers of lavageable macrophages decreased at 1 day and 1 month in response to LA. BALF macrophages also decreased in SH and SHHF at 1 day, but this effect was less remarkable than the effect in WKY. SH and SHHF exposed to 1 mg LA at 1 week had greater numbers of macrophages than controls when compared to dose-matched WKY. This increase in macrophages persisted only in SH through 1 month. The value of total cells in each of the three strains reflected changes in macrophages and neutrophils together.

Fe-binding proteins and capacities in BALF

One of the objectives of this study was to evaluate potential strain-related differences in nonheme Fe pool in response to acute LA exposure. Therefore, BALF and also lung tissue levels of ferritin and transferrin (Trf) at both the protein and transcriptional level, and Fe-binding capacities were analyzed. As we have noted in our previous study, the baseline (controls) BALF levels of ferritin and Trf were highly elevated in SH and SHHF when compared to WKY. BALF ferritin protein levels were found to be significantly elevated only in SH and SHHF exposed to LA in a concentration-dependent manner through 1 month (Figure 3). At 1 week and 1 month, SH, but not WKY or SHHF exposed to 1 mg LA, showed increases in

![Graphical representation of Figure 1: Temporal changes in BALF protein, albumin and lactate dehydrogenase, N-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyl transferase (GGT) activities in Wistar Kyoto (WKY), spontaneously hypertensive (SH) and spontaneously hypertensive heart failure (SHHF) rats following intratracheal instillation of saline (control), 0.25 mg Libby amphibole (LA) or 1 mg LA. Values are mean ± SE (WKY, n = 8–12/group; SH and SHHF, n = 6/group). *Significant difference within strain in respect to saline controls (p < 0.05). #Significant difference from WKY at the same exposure concentration (p < 0.05).]
BALF Trf relative to strain matched controls. The increases in Trf might reflect greater vascular protein leakage, as demonstrated by BALF protein leakage, since circulating levels of Trf, unlike ferritin, are high as it serves as a major systemic Fe transporter protein (Ghio et al., 1998; Anderson and Vulpe, 2009).

The BALF levels of nonheme Fe, as in the case of ferritin and Trf, were also significantly elevated at baseline (saline controls) in both SH and SHHF when compared to WKY. Nonheme Fe increased only in SH, but not in WKY or SHHF rats, in a concentration-dependent manner at 1 day and 1 week time points (Figure 3), which is associated with increases in vascular protein leakage. The nonheme Fe data at 1 month was highly variable within and between groups and inconsistent with other time points. We presumed that determination of airway lining Fe-binding capacity might allow one to understand the role of Fe movement between BALF Fe-binding proteins and inhaled fibers. We determined unsaturated and saturated (total) Fe-binding capacities (UIBC and TIBC) in BALF. Although the levels appeared to be high in SH and SHHF, no significant baseline strain differences existed in UIBC in this study (Figure 3). This is unlike our previous study where SH and SHHF rats had increased unsaturated Fe-binding capacities in BALF (Shannahan et al., 2010). The changes in BALF UIBC due to LA exposure were small and showed an increase, which was significant only in WKY at 1 month when compared to WKY saline control. In general, TIBC was found to be elevated in control SHHF compared to control WKY (Figure 3). LA exposure did not alter TIBC at any time point in any strain.

Lung expression of mRNA markers of Fe homeostasis, oxidative stress and inflammation

To understand the relationship between LA-induced pulmonary injury/inflammation and Fe homeostasis, we determined transcriptional levels of genes that regulate heme catabolism, Fe transport, Fe storage, oxidative stress and inflammation in rats exposed to saline or LA. As expected, the baseline expression of MIP-2 (a potent neutrophil chemoattractant), and HO-1 (a marker of oxidative stress) were markedly elevated in SH and SHHF relative to WKY, which supports the baseline differences in neutrophilic inflammation between the three strains. Surprisingly, only WKY increased MIP-2 in a concentration-dependent manner at 1 day in response to LA, despite neutrophilic inflammation being similar in all three strains at 1 day and persistent in SH and SHHF up to 1 month (Figure 4). No exposure-related induction of HO-1 expression was noted at 1 day in any of the strains; on the contrary, its expression was decreased from already high baseline by LA exposure in SHHF (Figure 4). At 1 week after 1 mg LA exposure, HO-1 expression was increased only in the SH rats. Because ferritin protein is made of FLC and FHC subunits, each possessing different
kinetic property for binding Fe, we determined transcriptional activation of both these subunits. FHC at all time points and FLC mRNA at 1 day and 1 week demonstrated no consistent strain-related baseline differences (saline controls); however, at 1 month the expression of FLC in SH and SHHF was greatly enhanced at baseline relative to WKY. These baseline alterations suggest a disease-associated progression in FLC expression. FLC mRNA was not induced after LA exposure in any strains at 1 day or 1 week, except for a small but significant rise in SHHF at 0.25 mg LA dose. However, at 1 month its expression increased in WKY as well as SH at high concentration of LA. Surprisingly, SHHF showed a significant decline in its expression from already increased baseline, suggesting that less compromised SH retained the capacity to sustain LA-induced FLC expression while highly compromised SHHF lost this ability. FHC mRNA expression was elevated in a concentration-dependent manner in only in SHHF at 1 day but not at later time points. No consistent strain or exposure-related differences were noted in Trf or DMT-1.

**Fe-binding proteins and capacities in lung tissue**

Lung ferritin protein also exhibited strain-related differences at baseline (SHHF > SH > WKY), which were unchanged 1 day after exposure to LA. At 1 week, however, only SH rats exposed to 1 mg LA had increased ferritin protein in lung tissue compared to saline controls (Figure 5). At 1 month no changes in ferritin levels were observed due to exposure in any strain. Lung Trf protein levels were generally higher at baseline in SH and SHHF rats. LA exposure did not affect Trf protein in the lung at any time in WKY and SHHF but resulted in a concentration-dependent increase at 1 day in SH rats (Figure 5). No major alterations were seen in lung tissue levels of non-Heme Fe, UIBC or TIBC (data not shown).

Figure 3. Time-related changes in markers of Fe homeostasis in the bronchoalveolar lavage fluid (BALF) of Wistar Kyoto (WKY), spontaneously hypertensive (SH) and spontaneously hypertensive heart failure (SHHF) rats intratracheally exposed to saline (control), 0.25 mg Libby amphibole (LA) or 1 mg LA. Unsaturated Fe-binding capacity (UIBC) is a measurement of the capacity of BALF components to bind catalytically active Fe. TIBC (total Fe-binding capacity), a calculated value (UIBC + nonheme Fe), is a measurement of all the Fe currently bound to BALF components. Values are mean ± SE (WKY, n = 8–12/group; SH and SHHF, n = 6/group). *Significant difference within strain in respect to saline controls (p < 0.05). #Significant difference from WKY at the same exposure concentration (p < 0.05).
Antioxidant levels in BALF and lung tissue

Alveolar Fe homeostasis and oxidative stress will likely be influenced by antioxidants such as ascorbate and glutathione in BALF and lung tissue. Baseline ascorbate levels in BALF were generally lower in SH and SHHF when compared to WKY (Figure 6). SH, and to some extent WKY and SHHF, exposed to 0.25 mg of LA at all time points exhibited a general decrease in BALF ascorbate when compared to their saline controls; however, most of these changes were insignificant except in SH at 1 day. There were marked variations in the baseline levels of lung tissue ascorbate among all three strains at each time point. Ascorbate in the lung tissue of SH was decreased due to exposure at 1 day, while WKY and SHHF demonstrate no marked changes. At 1 week lung tissue ascorbate levels were elevated in WKY in response to 0.25 mg LA exposure but unchanged in both SH and SHHF. WKY lung tissue ascorbate levels returned to baseline by 1 month while SH still demonstrated decreases after exposure. BALF glutathione was undetectable in all strains, while lung levels of glutathione were not found to be different between strains at baseline and remained unaltered due to LA exposure in all animal models (Figure 6).

Lung histopathology

Although the strain-related differences in specific biomarkers in lung and BALF were apparent, pathologically the LA-induced lesion severity differences could not be distinguished between strains. Marked histological alterations were noted in all three strains in time- and concentration-related manner following LA exposure (Table 1). At 1 day the types of lesions characterized
by acute alveolar neutrophilic inflammation and cell injury were LA concentration-dependent. The distribution of inflammatory foci in the lungs at 1 day was centriacinar but multifocal, and generally consisted of neutrophilic influx, associated with macrophages present within the lumen of the terminal bronchiole, alveolar duct and adjacent alveoli (Figure 7A, 7B and 7C). These changes were associated with minimal alveolar hyperplasia, in which the lining alveolar epithelium tended to be cuboidal instead of flattened. No incidences of interstitial fibrosis were observed at 1 day in any strain at either dose. However, at 1 week and at 1 month as shown in Figure 7E and 7F, all animals developed multifocal interstitial fibrosis and alveolar hyperplasia in a concentration-dependent manner (Table 1 and Figure 7D, 7E and 7F). By 1 month neutrophil-like cells were not readily apparent while macrophages persisted within alveoli and interstitium (Figure 7). In a few rats receiving 1 mg of LA focal granuloma-like formation was noted at this time. The severity scores
and incidence for each of these pathological indices are given in Table 1.

### Discussion

The ROS generation and toxicity of asbestos fibers has been shown to be increased by surface-complexed Fe and also by binding of tissue-associated Fe (Kamp et al., 1995b; Governa et al., 1999; Gazzano et al., 2007b). In this study, we postulated that genetically predisposed rat models with CVD-associated pulmonary Fe-overload would show greater pulmonary injury and inflammation following LA exposure when compared to healthy WKY rats due to increased availability of endogenous Fe to complex with LA. While initial inflammatory response to LA was only slightly exacerbated in SHHF, we found that there was a greater persistency of inflammation in SH and SHHF relative to WKY. This was associated with changes in Fe homeostasis biomarkers with a general increase in Fe-binding proteins only in SH and SHHF rats suggesting that more readily available endogenous protein-complexed Fe might be released over time to bind LA fibers thus causing persistency in inflammation. The formation of ferruginous bodies over asbestos fibers is known to occur with persistent inflammation over a long period of time in humans (Ghio et al., 2003).

MIP-2, which is a major neutrophil chemotactic factor in rats (Smartt et al., 2010), has been shown to be increased in epithelial and mesothelial cells in vitro, and in the pleural lavage of F344 rats following asbestos exposure (Driscoll et al., 1998; Hill et al., 2003). WKY were able to mount an acute and largely reversible inflammatory response (neutrophilic influx) with the reversible induction of MIP-2 mRNA, which was associated with activation of alveolar macrophages (increased NAG activity in BALF). However, SH and SHHF, despite their initial robust neutrophilic inflammation at 1 day, did not increase expression of MIP-2. Antioxidant deficiency in alveolar lining might result in earlier MIP-2 induction in SH and SHHF rats. Ascorbate, a key antioxidant of airway lining and the first line of defense, was lower at baseline in SH and SHHF and not increased following LA exposure.
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when compared to WKY. Early initial oxidant stress might have triggered MIP-2 expression prior to 1 day in SH and SHHF whereas it was delayed in WKY due to availability of sufficient ascorbate in the airway lining. Our subsequent studies have shown induction of MIP-2 at 4 h in SH rats following LA exposure (Shannahan et al., 2011).

Exposure to asbestos has been shown to cause acute pulmonary inflammation following intratracheal instillation and inhalation exposure in animals and has been postulated to contribute to chronic diseases (Fattman et al., 2006; Dostert et al., 2008). The predominance of neutrophilic inflammation acutely after LA exposure is expected based on the physicochemical nature of inhaled fibers as observed in earlier studies (Dorger et al., 2002; Putnam et al., 2008). In this study however, the mechanisms mediating this inflammatory response was likely different between healthy and Fe-overloaded CVD models because only WKY transiently increased MIP-2 mRNA in response to LA exposure at 1 day whereas only SH and SHHF demonstrated changes in Fe regulatory proteins. The observation that neutrophilic influx was more persistent in SH and SHHF compared to WKY exposed to LA suggests that they may be more sensitive to developing chronic active inflammation associated with lung disease.

Biomarkers of Fe homeostasis were significantly elevated at baseline and also changed in response to LA only in SH and SHHF relative to WKY. Specifically, the expression of FHC, which is considered important in sequestering Fe during acute inflammation/injury (Koorts and Viljoen, 2007) was induced only acutely and only in SHHF rats in an LA concentration-dependent manner which was accompanied by increases in BALF ferritin. FLC, which is known to be induced with chronic Fe-overload, was increased later in SH and SHHF at baseline consistent with its role in long-term baseline inflammation. However, unlike SH and WKY, the inability of SHHF to transcribe FLC in response to LA at 1 month when baseline expression is already very high suggests that more severe Fe-overload might impair their ability to further increase ferritin, which can lead to increases in the availability of catalytically active Fe and, thus, might contribute to chronic inflammation and injury following LA exposure. Increases in BALF ferritin were associated with an increased nonheme Fe and Trf in SHHF at baseline and also following LA exposure. These data further support the hypothesis that increased mobility of endogenous Fe from its protein-bound form in rat models of CVD-associated Fe-overload and their inability to maintain normal Fe

Figure 7. Time-related histological alterations in the lung following a single intratracheal instillation of Libby amphibole (LA) in Wistar Kyoto (WKY) rats. The photomicrographs for WKY rats 1 day and 1 month postexposure to saline, 0.25 and 1.0 mg LA are shown. A and D: Representative histological sections of lungs from WKY rats exposed to saline at 1 day and 1 month, respectively. B and E: Histological sections of lungs from WKY rats exposed to 0.25 mg LA at 1 day and 1 month, respectively. C and F: Histological sections of lungs from WKY rats exposed to 1.0 mg LA at 1 day and 1 month, respectively. Red arrows denote alveolar neutrophilic influx, green arrows show intraalveolar macrophage accumulation, blue arrows indicate alveolar hyperplasia, and black arrows interstitial fibrosis. All images are at ≥20 magnification. (See colour version of this figure online at www.informahealthcare.com/iht)
HO-1 is an inducible enzyme, which catabolizes heme into labile Fe, carbon monoxide and biliverdin (Driscoll et al., 1998, Gozzelino et al., 2010). The labile Fe released from HO-1 induces ferritin production, which binds and stores Fe, thereby reducing oxidative stress and thus plays an important role in the antioxidant response to Fe-mediated ROS generation. In our study, only SH rats exposed to LA were able to induce HO-1 mRNA expression at 1 week, which accompanied elevations in BALF and lung ferritin protein but not mRNA suggesting that transcriptional activation might have occurred in the course of 1 week. The increased baseline expression of HO-1 coincided with high baseline levels of ferritin protein and mRNA in SHHF rats. Interestingly, SHHF, already high in baseline expression, showed an inhibition of HO-1 expression at 1 day due to LA exposure. Since mice lacking the ability to induce HO-1 are more susceptible to the cytotoxic effects of ROS and inflammation (Poss and Tonegawa, 1997; Kapturczak et al., 2004), it is possible that the SHHF might be more vulnerable to injury and chronic exacerbation due to inhibition of HO-1 after LA exposure. However, acute transcriptional activation of FHC despite inhibition HO-1 due to LA exposure suggests a complex mechanism of regulating oxidative stress and Fe homeostasis in the SHHF.

Asbestos fibers are known to cause acute oxidative stress and inflammation that persists over time and contributes to chronic fibrosis, granuloma and lung cancer. We show the presence of inflammation at all times and fibrosis at 1 week and 1 month following a single dose of LA in all rat strains, which leads to focal granuloma formation in a few animals at 1 month. Although it is believed that environmentally relevant low-level exposures over a long period of time are not associated with lung inflammation, and fibrosis following asbestos exposure in humans, it has not been experimentally demonstrated. In animals, inflammation develops as soon as 3 days following inhalation of asbestosis at occupationally relevant concentrations (earliest determined) leading to pulmonary fibrosis over longer duration (Sabo-Attwood et al., 2005; Dostert et al., 2008). The persistence of inflammation as evidenced by increases in BALF cellularity and histological alterations suggests a persistent immune cell response, critical in chronic pulmonary disease, especially in rats with underlying Fe-overload.

There are limitations in generalizing the role of Fe-overload in LA-induced lung injury. The genetic rat models of CVD-associated Fe-overload may not exactly mimic the mechanisms of Fe-overload in humans. While intratracheal instillation allows us to precisely control the exposure dose, it will produce a one time bolus effect which may not be apparent with inhalation, where exposure continues with concurrent compensatory response. Because the fiber length in the LA mix used in this study is relatively shorter than other asbestos materials such as amosite and crocidolite, it is likely that the major portion of LA would have been phagocytosed by alveolar macrophages. Thus, the pattern of lung injury caused by LA could be very different from other fibers that are not phagocytosed. However, we have noted that, as in case of other fiber types, LA can bind exogenous Fe (Shannahan et al., 2011). Due to the difference in fiber characteristics, the kinetics of clearance of LA fibers might be different from other long fibers. The clearance of LA is also likely different in different between rat models and can influence the degree of lung response.

Conclusions
In conclusion, SH and especially SHHF demonstrated changes at baseline in markers of Fe homeostasis, oxidative stress and inflammation relative to WKY. Acute exposure to LA-induced transient pulmonary inflammation and MIP-2 expression in WKY without increases in markers of Fe homeostasis (ferritin) and oxidative stress (HO-1). However, in SHHF, and to some extent in SH, LA exposure caused more persistent inflammation over 1 week and 1 month, which was associated with marked changes in biomarkers of iron homeostasis with a generalized increase in ferritin. These findings suggest the involvement of endogenous Fe metabolism in more persistent pulmonary neutrophilic inflammation seen in SH and SHHF, which may be critical in their susceptibility to chronic asbestos-related disease.

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