Chrysotile induces ER stress in macrophages

Asbestos-induced disruption of calcium homeostasis induces endoplasmic reticulum stress in macrophages.

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Running Title: Chrysotile induces ER stress in macrophages

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Keywords: calcium, endoplasmic reticulum stress (ER stress), fibrosis, lung injury, macrophage, chrysotile, asbestos

Background: Macrophages are important cells in fibrotic diseases.

Results: Chrysotile increases cytosolic calcium (Ca^{2+}) and induces ER stress in macrophages in a Ca^{2+}-dependent manner. ER stress is found in alveolar macrophages from fibrotic lungs.

Conclusion: Chrysotile triggers ER Ca^{2+} leak and induces ER stress in macrophages.

Significance: Macrophages undergo ER stress, which may contribute to pulmonary fibrosis.

ABSTRACT
Though the mechanisms for fibrosis development remain largely unknown, recent evidence indicates that endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) may act as an important fibrotic stimulus in diseased lungs. ER stress is observed in lungs of patients with idiopathic pulmonary fibrosis. In this study we evaluated if ER stress and the UPR was present in macrophages exposed to chrysotile asbestos and if ER stress in macrophages was associated with asbestos-induced pulmonary fibrosis. Macrophages exposed to chrysotile had elevated transcript levels of several ER stress genes. Macrophages loaded with the Ca^{2+}-sensitive dye Fura-2-AM showed that cytosolic Ca^{2+} increased significantly within minutes after chrysotile exposure and remained elevated for a prolonged time. Chrysotile-induced increases in cytosolic Ca^{2+} were partially inhibited by either anisomycin, an inhibitor of passive Ca^{2+} leak from the ER, or BAPTA-AM, an intracellular Ca^{2+} chelator known to deplete ER Ca^{2+} stores. Anisomycin inhibited X-box binding protein 1 (XBP1) mRNA splicing and reduced immunoglobulin binding protein (BiP) levels, whereas BAPTA-AM increased XBP1 splicing and BiP expression, suggesting that ER calcium depletion may be one factor contributing to ER stress in cells exposed to chrysotile. To evaluate ER stress in vivo, asbestos-exposed mice showed fibrosis development, and alveolar macrophages from fibrotic mice showed increased expression of BiP. Bronchoalveolar macrophages from asbestosis patients showed increased expression of several ER stress genes compared to normal subjects. These findings suggest that alveolar macrophages undergo ER stress, which is associated with fibrosis development.

Aberrant repair of injured tissue is a characteristic feature of fibrotic remodeling, including pulmonary fibrosis. A prototypical type of pulmonary fibrosis is caused by asbestos exposure, which is prevalent worldwide as at least 125 million are exposed to hazardous levels, including 1.3 million workers in the U.S (1). The pathogenesis of asbestosis is not fully understood; however, recent evidence suggests that endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) may contribute to fibrosis development in a variety of tissues, including the lung (2,3).

The ER is involved in many cellular functions, including calcium (Ca^{2+}) storage and signaling and
the folding and maturation of proteins. Conditions such as ER Ca\(^{2+}\) depletion, oxidative stress, viral infections, glucose deprivation, and environmental exposures can impair ER function leading to accumulation of unfolded or misfolded proteins in the ER lumen (4,5). Cells respond to ER stress by activating a homeostatic signaling network, the UPR. The UPR is designed to attenuate ER stress and consists of three adaptive signaling pathways that act to restore ER protein folding capacity via: 1) decreasing protein load by inhibiting translation; 2) increasing ER chaperone proteins by transcriptional up-regulation; and 3) increasing ER-associated protein degradation of unfolded proteins. The UPR includes three ER resident transmembrane proteins, inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) that act as sensors by monitoring ER protein folding status. Under basal conditions, these proteins are bound by the ER chaperone BiP (immunoglobulin binding protein or glucose-regulated protein 78-kDa, GRP78) and are maintained in an inactive state. When ER stress develops and unfolded proteins accumulate, BiP is released from IRE-1, PERK and ATF6, which triggers activation of the downstream pathways of the UPR. While the UPR is designed to alleviate ER stress, prolonged or severe UPR activation can modulate inflammation and initiate programmed cell death contributing to the pathogenesis of various human diseases (4-6).

ER stress in alveolar type II cells (AEC) has been shown to be present in fibrotic loci in patients with pulmonary fibrosis (7,8). ER stress in AECs can be induced by mutations in surfactant protein C (SPC) (8,9), mutations in SPA (10), and chronic herpes virus infection (9,11), and ER stress can result in intrinsic apoptosis secondary to mitochondrial dysfunction (12). UPR markers and increased collagen Type I expression have been observed in fibroblasts of fibrotic lungs, a response likely mediated by activation of TGF-\(\beta\) signaling (7). Alveolar macrophages have an important role in fibrosis development (13-15); however, the role of ER stress in macrophages in pulmonary fibrosis has not been investigated.

Depletion of ER Ca\(^{2+}\) stores can induce ER stress (16). Thapsigargin (Tg), a strong inducer of ER stress, selectively inhibits the ER Ca\(^{2+}\)-ATPase (17), which reduces active transport of Ca\(^{2+}\) into the ER lumen resulting in increases in cytosolic Ca\(^{2+}\) (18) as well as gradual depletion of ER Ca\(^{2+}\) stores. Because BiP and other chaperones are Ca\(^{2+}\)-binding proteins, ER Ca\(^{2+}\) depletion impairs chaperone activity resulting in accumulation of unfolded proteins and activation of an ER stress response. Recent studies using Tg in combination with Ca\(^{2+}\) chelator BAPTA-AM to deplete ER Ca\(^{2+}\) stores have shown induction of the UPR in endothelial cells (19) and PC12 cells (20). Notably, ER Ca\(^{2+}\) leak through the translocon has recently revealed as a possible mechanism for ER Ca\(^{2+}\) depletion leading to activation of the UPR (21,22).

Disruption of Ca\(^{2+}\) homeostasis has not been directly linked to fibrotic conditions, and the role of Ca\(^{2+}\) depletion and ER stress in macrophages in the development of pulmonary fibrosis is not known. The objective of this study was to determine if chrysotile asbestos elicits ER stress secondary to alterations ER Ca\(^{2+}\) release in macrophages. To provide biological relevance, we determined if alveolar macrophages exhibit ER stress markers in chrysotile-exposed fibrotic mice and in patients with asbestosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chrysotile asbestos was provided by Dr. Peter S. Thorne of the University of Iowa College of Public Health. Chrysotile stock solutions (10 mg/ml) were prepared in PBS without calcium or magnesium, stored at -20°C, and vortexed vigorously before use. Fura2-AM, Fluo-3 AM, and ionomycin were from Invitrogen. Rabbit BiP/GRP78 antibody was obtained from Cell Signaling, and mouse monoclonal ATF6 antibody and FCCP (carbonyl cyanide-p- trifluoromethoxyphenylhydrazone) were from abcam. Human inositol 1,4,5-triphosphate receptor (IP3R-1) siRNA was from Santa Cruz Biotechnology, Inc. \(\beta\)-actin antibody, thapsigargin (Tg) and tunicamycin (TM) were obtained from Sigma, and ansiomycin was from Millipore. The mouse transforming growth factor beta-1 (TGF-\(\beta\)1) Duo Set Elisa kit was obtained from R&D Systems, Inc.

**Human Subjects**—The Human Subjects Review Board of the University of Iowa Carver College of Medicine approved the protocol of obtaining alveolar macrophages from normal volunteers and patients with asbestosis. Normal
volunteers had to meet the following criteria: (1) age between 18 and 55 years; (2) no history of cardiopulmonary disease or other chronic disease; (3) no prescription or nonprescription medication except oral contraceptives; (4) no recent or current evidence of infection; and (5) lifetime nonsmoker. Alveolar macrophages were also obtained from patients with asbestosis. Patients with asbestosis had to meet the following criteria: (1) FVC and DLCO at least 50% predicted; (2) current nonsmoker; (3) no recent or current evidence of infection; and (4) evidence of restrictive physiology on pulmonary function tests and interstitial fibrosis on chest computed tomography.

Fiberoptic bronchoscopy with bronchoalveolar lavage was performed after subjects received intramuscular atropine (0.6 mg) and local anesthesia. Three subsegments of the lung were lavaged with five 20 ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of alveolar macrophages was determined by Wright-Giemsa stain and varied from 90 to 98%.

Mice—C57BL/6J mice were purchased from Jackson Laboratories. All protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. Mice were anesthetized with 3% isoflurane using a precision Fortec vaporizer (Cyprane, Keighley, UK) and then given either 125 µg titanium dioxide (TiO2) or 125 µg of chrysotile (Chry) asbestos in 75 µL of 0.9% saline by the intratracheal route. Mice were euthanized 21d after chrysotile exposure, a time interval that elicits histological and biochemical evidence of pulmonary fibrosis in mice (14). Bronchoalveolar lavage (BAL) was performed and BAL cells isolated for differential cell counts and for RNA isolation. Lungs were removed and fixed in formalin for assessment of collagen deposition using Masson’s trichrome staining and by hydroxyproline assay.

Cell Culture—Human monocytic THP-1 cell line was obtained from American Type Culture Collection. Cells were maintained in RPMI-1640 medium containing 10 mM HEPES, 1 mM sodium pyruvate, 2.5 g/L glucose, 2 mM L-glutamine, 10% fetal bovine serum, and penicillin/streptomycin. For experiments, THP1 cells (~1 X 10⁶ cells/ml) were incubated in RPMI medium, phenol red free, containing 0.5% fetal bovine serum. Bone marrow-derived macrophages (BMDM) were isolated from C57BL/6J mice femur and tibia bones according protocol described by Weischenfeldt and Porse (23). Briefly, isolated BMDM were grown in culture dishes in DMEM medium containing 10% fetal bovine serum, penicillin/streptomycin, and supplemented with 10% L929 conditioned medium as a source of macrophage colony-stimulating factor (M-CSF). Cells were grown for 6-7 d and then plated in 6-well dishes at a density of ~1.5 X10⁶ cells/well for an additional 24h. Cells were cultured in phenol red free RPMI medium containing 0.5% fetal bovine serum for experiments.

Immunoblot Analysis—Cell protein lysates were harvested in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl and 1% Nonidet P-40) containing a protease inhibitor mix (Roche, Complete Mini tablets) and a phosphatase inhibitor mix (Calbiochem no. 524625). Cell lysates were sonicated for 30 s and cleared by centrifugation (10,000 rpm at 4°C). Supernatants were assayed for protein content using a DC™ Protein Assay kit (Bio-Rad). Lysates (10-50 µg) were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblot analysis conducted as described (24). Primary antibody dilutions used for GRP78/BiP (1:1000), ATF6 (1:1000) and β-actin (1:40,000) were followed by the appropriate secondary antibody crosslinked to horseradish peroxidase. Protein bands were visualized by enhanced chemiluminescence (Amersham ECL Prime) and quantified by densitometry using ImageJ Software (nih.gov).

Real-time RT-PCR—Quantitative RT-PCR was conducted by isolating total RNA using TRIzol Reagent (Life Technologies) and synthesizing cDNA from 1-2 µg of RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). PCR was conducted using iQ™ SYBR® Green Supermix (Bio-Rad) with target cDNA and 15 pmol of reverse and forward gene-specific primers as follows: 3 min at 95 °C, then 45 cycles of 95 °C 20 s, 60 °C 20s and 72 °C 20s. Amplification specificity was confirmed by a melting curve analysis. Sample mRNA abundance was calculated using the cycle threshold (ΔΔCT) method. The relative expression of each gene was normalized to the quantity of hypoxanthine-guanine phosphoribosyltransferase or β-actin.
Chrysotile induces ER stress in macrophages

Gene specific primers were designed using the NCBI sequence database and were purchased from Integrated DNA technologies (Coralville, Iowa).

**XBP1 mRNA Splicing Assay**—Splicing of X-box binding protein 1 (XBP1) mRNA is used as a measure of IRE-1 activation. Conventional RT-PCR was conducted to detect the presence of XBP1 mRNA splicing (25). Total RNA was isolated and cDNA synthesized as above described. The following primers were used to amplify both unspliced and spliced human XBP1 mRNA: XBP1-F, 5'- TTA CGA GAG AAA ACT CAT GGC C -3'  and XBP1-R, 5'- GGG TCC AAG TTG TCC AGA ATG C -3' to yield products of 289 bp and 263 bp, respectively. Primers used for unspliced and spliced mouse XBP1 mRNA: GAA CCA GGA GTT AAG AAC ACG  and AGG CAA CAG TGT CAG AGT CC  to yield products of 205 and 179 bp, respectively. PCR was conducted using target cDNA with Phusion DNA polymerase (New England Biolabs) as follows: 2 min at 98°C, then 35 cycles of 98°C 20s, 52°C 30s, 72°C 20s followed by 1 min at 72°C. PCR products were separated in a 10% acrylamide gel and the bands visualized by silver staining.

**Measurement of cytosolic Ca^{2+}**—Human THP1 cells were loaded with Fura2-AM (1 µM, 30 min, 37°C) at a cell density of 1 X 10^6 cells/ml in RPMI medium. Cells were washed twice in Hanks Balanced Salt Solution (HBSS, Gibco Life Technologies) containing 1.3 mM CaCl_2 or Ca^{2+}-free HBSS. HBSS had the base composition of 138 mM NaCl, 1.0 mM MgCl_2, 5.3 mM KCl, 4.2 mM NaHCO_3, 0.45 mM KH_2PO_4, 0.33 mM Na_2HPO_4, pH 7.3, and 5.6 mM glucose. After washing, cells were suspended in Ca^{2+}-containing or Ca^{2+}-free HBSS and incubated a further 30 min to allow for acetoxyethyl (AM) esterase cleavage. In some experiments, cells were loaded with BAPTA-AM (1-5 µM, 30 min, 37°C) using the same procedures. Suspended cells were loaded into a 96 well plate (~1.75 X10^5 cells/well) and placed into a SpectroMax M2 plate reader (Molecular Devices) set at 37°C for fluorescence measurements. Fura2 dye was excited through 340 nm and 380 nm filters and fluorescence emission collected at 510 nm. Data collection at 1-10 min intervals was performed using SoftMax Pro 6.1 software (Molecular Devices). The ratio of fluorescence intensity of 340nm/380nm (F340/F380) was used to determine intracellular free calcium.

**Confocal Microscopy**—THP1 cells were loaded with Fluo-3 AM (2.5 µM, 60 min, 37°C), washed twice in HBSS, suspended in RPMI medium 1640 containing 0.5% bovine serum, and placed in chambered coverslips. Fluorescence images were collected using a Zeiss 510 LSM Confocal Microscope. Fluo-3 dye was excited at 488 nm and emission collected by a 475-500 nm filter.

**siRNA Transfection**—siRNA against human IP3R-1 was from Santa Cruz Biotechnology, Inc. (sc-42475) and described as a pool of three target specific 19-25 nt siRNA designed to knock down human IP3R-1 gene expression. The IP3R-1 siRNA (100 nM) was transfected into THP1 cells (~0.5 X 10^6 cells/ml) using DharmaFECT 2 transfection reagent (Dharmacon Research, Inc.) according to manufacturer’s instructions. After 6h of transfection, the medium was switched to RPMI-1640 medium containing 10% FBS, and 48h later, the indicated experiments were conducted.

**Hydroxyproline Assay**—Lung tissue was dried at 100°C to a stable weight and hydrolyzed with 6N HCl for 24h at 112°C. Lung hydroxyproline content was determined as described (14) and expressed relative to lung dry weight.

**Statistical Analysis**—A Student’s unpaired, two-tailed t test was used to assess statistical differences between two groups. A one-way ANOVA was used when comparing more than two groups with Tukey post hoc test to determine differences. Data were expressed as mean±sem. Probability values p < 0.05 were considered significant.

**RESULTS**

**Chrysotile induces ER stress in macrophages**—To determine if chrysotile induces an ER stress response in macrophages, we first examined the IRE/XBP1 pathway. Activation of the IRE1/XBP1 pathway results in the splicing of a 26 bp fragment from the mRNA encoding transcription factor XBP1. XBP1 mRNA splicing generates an active XBP1 transcription factor that acts as a potent inducer of select ER stress genes. Macrophages exposed to chrysotile showed splicing of XBP1 mRNA after 24h, but not at earlier time intervals (Fig. 1A). In contrast,
Chrysotile induces ER stress in macrophages

Thapsigargin (Tg, 100 nM), an ER Ca\(^{2+}\)-ATPase inhibitor used as a positive control, elicited significant XBP1 splicing within 3-6 h.

BiP is known as a master regulator of the ER stress response (6), thus we evaluated the expression of BiP as well as the ER stress transcriptional enhancer ATF6\(\alpha\) by immunoblot analysis. Macrophages exposed to chrysotile for 24 h and 48 h showed increased levels of BiP as well as elevated ATF6\(\alpha\) (Fig. 1B). Densitometric quantification of immunoblots revealed an increase in normalized BiP protein in macrophages exposed for 24 h to chrysotile or Tg (Fig. 1C). We assessed macrophage BiP mRNA expression by quantitative real-time RT-PCR after chrysotile exposure and found increased BiP mRNA levels at 24 h and 48 h, but not at earlier times (3-6 h) after exposure (Fig. 1D). We also examined mRNA expression of other chaperone genes, and found that glucose-regulated protein (GRP)94, endoplasmic-resident protein (Erp)72, and protein kinase inhibitor 58kDa (P58IPK) transcripts were increased after 24 h chrysotile exposure (Fig. 1E-G). Notably, these ER stress genes are modulated, at least in part, by ATF6\(\alpha\) (26, 27).

Taken together, these results demonstrate activation of the ER stress response in macrophages exposed to chrysotile.

To determine if chrysotile elicited ER stress in primary macrophage cells, mouse bone marrow-derived macrophages (BMDM) were isolated and treated with the ER Ca\(^{2+}\)-ATPase inhibitor, Tg (100 nM) or with an inhibitor of N-linked glycosylation, tunicamycin (Tm, 5 \(\mu\)g/ml). BMDM exposed to chrysotile, Tg, or Tm for 24 h showed splicing of XBP1 mRNA (Fig. 2A) as well as increased levels of BiP protein (Fig. 2B-C), BiP mRNA (Fig. 2D), and DNA-damage inducible protein (CHOP) mRNA (Fig. 2E). Because current evidence suggests that ER stress can act as a pro-fibrotic stimulus (3) and because transforming growth factor beta-1 (TGF-\(\beta\)) can induce ER stress in lung fibroblasts (7), we asked if pro-fibrotic gene expression was detected in macrophages under ER stress. Interestingly, we found increased levels TGF-\(\beta\)1 mRNA in BMDM cells exposed to chrysotile or Tg, whereas TGF-\(\beta\)1 transcript levels remained unchanged in cells exposed to Tm for 24 h (Fig. 2F). These findings support the idea that chrysotile elicits ER stress in macrophages and, further, that increased expression of pro-fibrotic TGF-\(\beta\)1 is associated with ER stress in these cells.

Chrysotile-induced alterations in calcium flux are linked to the ER stress response—Because asbestos-induced Ca\(^{2+}\) release has been associated with ER stress in AECs (12) and since ER Ca\(^{2+}\) depletion has been linked to ER stress (16), we determined if Ca\(^{2+}\) release occurred in macrophages exposed to chrysotile. For our initial assessments, cells were loaded with Fluo-3-AM and evaluated by confocal microscopy. Chrysotile exposure increased cytosolic Ca\(^{2+}\) (Fig. 3A). In subsequent experiments, cells were loaded with Fura2-AM and exposed to chrysotile to evaluate time-dependent changes in Ca\(^{2+}\) release. Cytosolic Ca\(^{2+}\) levels were significantly increased above controls and remained elevated for the duration of the experiment (Fig. 3B). Addition of ionomycin, a Ca\(^{2+}\) ionophore, stimulated further increases in cytosolic Ca\(^{2+}\) levels (Fig. 3B), whereas addition of EDTA, a Ca\(^{2+}\) chelator, dramatically reduced cytosolic Ca\(^{2+}\) (Fig. 3C). When cells were exposed to chrysotile in Ca\(^{2+}\)-free medium, cytosolic Ca\(^{2+}\) increased, implicating the ER as an important source for Ca\(^{2+}\) in these cells (Fig. 3D). When CaCl\(_2\) was added, further increases in cytosolic Ca\(^{2+}\) were observed (Fig. 3D).

Because ER Ca\(^{2+}\) depletion and ER stress have been induced in cells loaded with the intracellular Ca\(^{2+}\) chelator, BAPTA-AM (20), we examined the effects of BAPTA-AM in macrophages exposed to chrysotile. Chrysotile steadily increased cytosolic Ca\(^{2+}\) levels in a time-dependent manner to values 5-6 fold above control values (Fig. 3E). However, BAPTA-AM reduced cytosolic Ca\(^{2+}\) levels below control values, and also inhibited the chrysotile-induced increases in cytosolic Ca\(^{2+}\) throughout the 180 min chrysotile exposure. During the final minutes of incubation, we added the mitochondrial uncoupler FCCP and observed sharp increases in cytosolic Ca\(^{2+}\), a response indicating cells were viable and actively regulating cellular Ca\(^{2+}\) before FCCP exposure (Fig. 3E). To determine if BAPTA-AM induces ER stress in macrophages, we loaded cells with BAPTA-AM and incubated cells for 24 h. BAPTA-AM elicited XBP1 splicing (Fig. 3F) and increased BiP protein (Fig. 3G) and BiP mRNA levels (Fig. 3H). Collectively, these findings demonstrate that chrysotile elicits sustained increases cytosolic Ca\(^{2+}\) levels, and
suggests that depletion of intracellular Ca\(^{2+}\) stores is linked to ER stress in macrophages.

Recent evidence indicates that ER calcium depletion during ER stress may occur by Ca\(^{2+}\) leak through the translocon, an ER protein complex involved in translation (21,22). Further, an inhibitor of translocon Ca\(^{2+}\) leak, anisomycin, has been shown to alter the ER stress response in certain cell types (28). To assess if anisomycin modulates Ca\(^{2+}\) flux in macrophages, Fura2-AM-loaded cells were pretreated with anisomycin before chrysotile exposure. As anticipated, anisomycin inhibited the chrysotile-induced increases in cytosolic Ca\(^{2+}\) in macrophages suspended in 1.3 mM Ca\(^{2+}\) medium (Fig. 4A) and in Ca\(^{2+}\) free medium (Fig. 4B). Anisomycin also inhibited BiP protein expression (Fig. 4C-D), BiP mRNA levels (Fig. 4E) as well as XBP1 mRNA splicing (Fig. 4F). In aggregate, these data suggest that inhibition of the translocon reduces chrysotile-induced ER Ca\(^{2+}\) release and attenuates the ER stress response.

To further evaluate how chrysotile-induced ER Ca\(^{2+}\) release is related to the ER stress response in macrophages, we conducted comparative experiments examining cytosolic Ca\(^{2+}\) levels in cells exposed to chrysotile or cells exposed to ER stress inducing agents Tg and TM. We first examined cytosolic Ca\(^{2+}\) levels in cells acutely exposed to chrysotile, Tg, or TM and found that chrysotile elicited prolonged increases cytosolic Ca\(^{2+}\) compared to transient elevations in cytosolic Ca\(^{2+}\) following the Tg or TM treatments (Fig. 5A-B). Notably, after 150 min of exposure, the addition of 2 mM CaCl\(_2\) triggered rapid and relatively large increases in cytosolic Ca\(^{2+}\) in cells treated with Tg compared to control or chrysotile or TM treated cells (Fig. 5B). This finding suggested that Tg treatment caused some degree of ER Ca\(^{2+}\) depletion and thereby activated store-operated Ca\(^{2+}\) channels (29) within this time frame.

Because ER Ca\(^{2+}\) depletion induced ER stress, we next determined if ER stress modulated Ca\(^{2+}\) release in cells under ER stress. Cells were treated with chrysotile or Tg or TM for 24h and assayed for Ca\(^{2+}\) release using the Ca\(^{2+}\) ionophore, ionomycin. Ionomycin-releasable Ca\(^{2+}\) was greater in cells previously treated with chrysotile, Tg or TM compared to control cells (Fig. 5C-D). Similar experiments were conducted to assess ER Ca\(^{2+}\) stores by asking if store-operated Ca\(^{2+}\) channels could be activated in cells under ER stress. As shown (Fig. 5E-F), addition of 2 mM CaCl\(_2\) to cells previously exposed to chrysotile, Tg or TM resulted in greater increases in cytosolic Ca\(^{2+}\) compared to control cells. Collectively, these findings suggest that macrophages under ER stress may exhibit increased ionomycin-releasable Ca\(^{2+}\) stores, yet these cells may still exhibit some degree of ER Ca\(^{2+}\) depletion.

Because the inositol 1,4,5-triphosphate receptor (IP\(_3\)R) is an IP\(_3\)-gated channel that releases Ca\(^{2+}\) from the ER, and because modulation of IP\(_3\)R activity is considered important for life or death decisions made by cells under ER stress (30), we asked if knockdown of IP\(_3\)R-1, the major IP\(_3\)R isoform present in macrophages (31), would alter cytosolic Ca\(^{2+}\) levels in macrophages exposed to chrysotile. We found that cells transfected with IP\(_3\)R-1 siRNA showed significantly reduced IP\(_3\)R-1 mRNA levels (Fig. 5G); however, cytosolic Ca\(^{2+}\) levels were not altered by chrysotile exposure (Fig. 5H).

Expression of the UPR in macrophages in vivo and ex vivo—Most prior studies have documented ER stress with activation of the UPR in AECs or in fibroblasts using various disease models (3,32), while ER stress in alveolar macrophages from diseased lung has not been investigated. To evaluate ER stress in macrophages in vivo, C57BL/6 mice were exposed either to TiO\(_2\) or chrysotile intratracheally and euthanized 21d later. The lungs of mice exposed to TiO\(_2\) were essentially normal. In contrast, chrysotile-exposed lungs showed destruction of normal lung architecture by widespread collagen deposition (Fig. 6A). The histological findings were confirmed biochemically by elevated hydroxyproline content in chrysotile-exposed lung tissue compared to TiO\(_2\)-exposed lungs (Fig. 6B). BAL samples from the two groups contained similar numbers of cells (~8.9 ± 4.4 X 10\(^5\) cells/mouse), and the predominant cells were alveolar macrophages obtained from fibrotic mice showed elevated BiP protein compared to BAL cell lysates from TiO\(_2\) mice (Fig. 6D-E). Quantitative RT-PCR from whole lung and BAL cells revealed elevated BiP transcript levels in BAL alveolar macrophages from chrysotile-exposed mice, but not in whole lung tissue in mice exposed to...
Chrysotile induces ER stress in macrophages

Because ER stress has been linked to increased apoptosis in AEC (8,9,11,12) in fibrotic lung, we determined if ER stress was associated with increased apoptosis in mouse BAL cells. There was no difference in apoptosis in alveolar macrophages from chrysotile- or TiO2-exposed mice, as measured by caspase-3 (Fig. 6G-H). To link ER stress to the fibrotic response following chrysotile exposure, we measured active TGF-β1 in BAL fluid and found that it was elevated in fibrotic mice compared to the TiO2-exposed mice (Fig. 6I). In aggregate, these data suggest that the UPR induces cell survival in macrophages and that macrophage survival is an important determinant of fibrosis development.

To support findings from fibrotic mice, BAL cells from normal subjects and asbestosis patients were evaluated for ER stress. Consistent with our in vitro and in vivo findings, BAL cell lysates from asbestosis patients showed increased levels of BiP protein as well as elevated ATF6α (Fig. 7A). Densitometric analysis of immunoblots revealed a significant increase in BiP protein (Fig. 7B) as well as ATF6α (Fig. 7C) in alveolar macrophages from asbestosis patients compared to normal controls. We also found that alveolar macrophages from asbestosis patients had an increase in BiP mRNA, endoplasmic oxidase 1-α (ERO1-α), and DNA-damage inducible protein (CHOP) mRNA levels compared to normal subjects (Fig. 7 D-F). In aggregate, these observations suggest that ER stress in alveolar macrophages is linked to the asbestos-induced pulmonary fibrosis and alleviation of ER stress may be a novel target for therapy.

DISCUSSION

ER stress and the UPR are key pathogenic events in disease processes as different as atherosclerosis, heart disease, diabetes, cancer, and pulmonary fibrosis (4-6,33). Regarding fibrosis, most available evidence indicates that ER stress enhances the vulnerability of structural cells such as AECs and fibroblasts to fibrotic stimuli. In these studies, ER stress and UPR in AECs has been associated with herpes virus infection (9,11), altered surfactant protein processing (9), expression of mutant surfactant proteins (9,10,34,35), apoptosis (8,11,12), epithelial-mesenchymal transitions (7), and induction of an inflammatory response (35) that ultimately leads to a profibrotic environment in lung tissue (2,3). To our knowledge, no studies have examined the UPR in macrophages in the setting of lung fibrosis.

The objective of this study was to investigate ER stress in alveolar macrophages exposed to chrysotile and to link ER Ca2+ release to induction of ER stress. Similar to a prior study using A549 cells (12), chrysotile asbestos exposure triggered rapid and sustained increases in cytosolic Ca2+ within minutes and increased ER stress markers (IRE1/XBP1 mRNA splicing, GRP78/BiP, ATF6α) within 24h. Chrysotile-induced increases in cytosolic Ca2+ were largely inhibited by anisomycin, an inhibitor of passive Ca2+ leak from the ER (22), or BAPTA-AM, a Ca2+ chelator known to deplete ER Ca2+ stores (20). Anisomycin inhibited induction of ER stress markers, whereas BAPTA enhanced ER stress, results suggesting that ER calcium depletion may be one factor contributing to ER stress in macrophages exposed to chrysotile.

ER Ca2+ stores are essential for protein folding as well as Ca2+ signaling, and thus ER Ca2+ depletion may act as an important upstream event in the pathogenesis of many diseases (16). Under basal and stimulated conditions, ER Ca2+ stores are dependent on many cellular processes, including mitochondrial ATP production, store-operated Ca2+ influx, ER Ca2+-ATPase activity, and ER Ca2+ leak. Presumably, impairments in any of these cellular processes can contribute to ER stress in macrophages exposed to chrysotile. Our findings indicate that external sources as well as the ER Ca2+ stores are important for Ca2+ release in cells exposed to chrysotile. Because we found that chrysotile elicits prolonged ER Ca2+ release, we hypothesized that ER Ca2+ depletion modulates ER stress in macrophages.

Recently, ER Ca2+ leak through the translocon, an ER protein complex involved in translation, has been shown to be a possible site for ER Ca2+ depletion leading to activation of the UPR (21,22). Translocons are protein-conducting channels found on the surface of rough ER. When ribosome-translocon complexes are free of polypeptide chains they remain open and conduct both ions and neutral molecules (36). To evaluate Ca2+ leak through the translocon, we used anisomycin (an inhibitor of peptidyltransferase) to...
Chrysotile induces ER stress in macrophages

maintain the ribosome-bound translocon in a closed state that reduces permeability to \( \text{Ca}^{2+} \) (22). We found that anisomycin partially inhibited chrysotile-induced increases in cytosolic \( \text{Ca}^{2+} \) in macrophages incubated in 1.3 mM \( \text{Ca}^{2+} \) and in \( \text{Ca}^{2+} \)-free media. These findings are consistent with studies that showed inhibition of the translocon reduced \( \text{Tg} \)-induced ER \( \text{Ca}^{2+} \) release in cells incubated in \( \text{Ca}^{2+} \)-free media (21,28) and support the idea that ER \( \text{Ca}^{2+} \) leak occurs in cells exposed to chrysotile.

ER \( \text{Ca}^{2+} \) leak may not be the only factor contributing to ER \( \text{Ca}^{2+} \) release and depletion in chrysotile-exposed macrophages. Oxidative stress can trigger ER stress and promote ER \( \text{Ca}^{2+} \) release (18), perhaps by inhibiting ER \( \text{Ca}^{2+} \)-ATPase activity (37). Alternatively, inositol 1,4,5-triphosphate receptor (\( \text{IP}_3\text{R} \)) is an \( \text{IP}_3 \)-gated channel that releases \( \text{Ca}^{2+} \) from the ER and is activated by \( \text{IP}_3 \), \( \text{Ca}^{2+} \), and by oxidative stress (30). We found that knockdown of \( \text{IP}_3\text{R}-1 \) did not alter chrysotile-induced increases in cytosolic \( \text{Ca}^{2+} \); however, our \( \text{Ca}^{2+} \) measurements were obtained from a population of cells, which may not provide the resolution necessary for detecting changes in \( \text{Ca}^{2+} \) within a single cell or within mitochondria (31,38). \( \text{IP}_3\text{R}-1 \) has been shown to be important for transferring \( \text{Ca}^{2+} \) directly to the mitochondria (38). A recent study in macrophages provided evidence for an UPR-CHOP - ERO1-\( \alpha \) pathway triggering \( \text{IP}_3\text{R}-1 \)-mediated \( \text{Ca}^{2+} \) release and apoptosis (31). Although our data suggest the involvement of the translocon and ER \( \text{Ca}^{2+} \) depletion in mediating ER stress, these observations do not exclude the contribution(s) of other mechanisms, including a possible role for \( \text{IP}_3\text{R}-1 \).

The current study demonstrates that BiP expression was elevated in alveolar macrophages obtained from human asbestosis patients and from mice with a fibrotic phenotype. BiP is known as the master regulator of the UPR (6), and BiP protein up-regulation is often used as an indicator of ER stress (39). To date the only known mechanism for activation of IRE1, PERK, or ATF6 is their release from BiP, although the mechanisms behind the differential activation of the different arms of the UPR are not fully understood (40). We found that chrysotile exposure increased expression of various chaperone genes (ERp72, GRP94, P58IPK) as well as other ER stress markers including ERO1-\( \alpha \) and CHOP. Prior studies have evaluated the role of ER stress in inducing apoptosis in AEC as a potential mechanism in the pathogenesis of pulmonary fibrosis (8,9,11,12,34). Our findings clearly reveal ER stress in chrysotile-exposed macrophages; however, we found no significant apoptosis in alveolar macrophages from chrysotile-exposed mice. As previously shown, these observations suggest that ER stress in macrophages exposed to chrysotile induces cell survival (38,41,42). Several chronic diseases have demonstrated that macrophage cell survival has an important role in disease progression (43-46). Our observations suggest that the UPR induce cell survival in macrophages, which mediates fibrosis development in lung. Moreover, the presence of ER stress in alveolar macrophages from fibrotic mice is significant in that lung homogenates did not exhibit elevated BiP expression suggesting that macrophages are an important mediator of the fibrotic response.

Stimulated macrophages display many different functions thought to be important for the pathogenesis of many disease states (47). Recent evidence indicates that alternatively activated (M2) macrophages are abundant in atherosclerotic lesions (48), and induction of ER stress induces macrophage polarization from the M1 into the M2 phenotype leading to increased cholesterol deposition and enhanced foam cell formation (49). Similar to the pro-inflammatory state induced by ER stress in AECs (35), ER stress in macrophages has been associated with pro-inflammatory effects though activation of both a JNK-TNF\( \alpha \) pathway and a CHOP-ERK-IL-6 pathway (50). When exposed to chrysotile, macrophages produce pro-fibrotic cytokines such as TGF-\( \beta \) as well as high levels of reactive oxygen species (ROS), including H\(_2\)O\(_2\) (13-15). In a recent study, we found that alveolar macrophages from asbestosis patients produce high levels of H\(_2\)O\(_2\), and these cells demonstrate a predominantly pro-fibrotic M2 phenotype. Moreover, polarization to the M2 phenotype was driven, in part, by Cu,Zn-SOD-mediated H\(_2\)O\(_2\) production in chrysotile-exposed cells (13). Our new observations demonstrate that chrysotile-exposed macrophages exhibit rapid and sustained increases in \( \text{Ca}^{2+} \) release and later develop ER stress. More importantly, alveolar macrophages obtained from asbestosis patients or
Chrysotile induces ER stress in macrophages from mice with a fibrotic phenotype exhibit ER stress. Because the predominance of M2 macrophages are linked to the development of fibrosis, it will be interesting in future studies to alter ER stress in vivo and investigate polarization of macrophages and its relationship to fibrosis.

ACKNOWLEDGEMENTS

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Chrysotile induces ER stress in macrophages

REFERENCES


Chrysotile induces ER stress in macrophages


Chrysotile induces ER stress in macrophages

Chrysotile induces ER stress in macrophages

FIGURE LEGENDS

FIGURE 1. Chrysotile induces ER stress in macrophages. (A) Macrophages were exposed to thapsigargin (Tg, 100 nM) or chrysotile (10 µg/cm²) for indicated times. Spliced XBP1 evaluated by conventional RT-PCR followed by acrylamide gel electrophoresis. (B) Macrophages exposed to chrysotile (10 µg/cm²) for 24h or 48h and cell lysates were used for glucose-regulated protein (GRP) 78/BiP and ATF6 expression by protein immunoblot analysis. (C) Densitometric analysis of BiP protein expression corrected for β-actin in cells exposed to chrysotile (Chry) or Tg for 24h. * or **p<0.05 versus control or Tg or Chry. n=8/group. Insert shows BiP immunoblot for 24h exposure. (D) Quantitative RT-PCR for BiP mRNA corrected for HPRT mRNA. Macrophages treated with chrysotile for indicated times or Tg for 24h. * or **p<0.05 versus other groups. n = 8/group. (E,F,G) Quantitative RT-PCR data showing mRNA of different ER stress genes corrected for HPRT mRNA. n = 3/group. RNA was isolated from macrophages 24h after exposure to chrysotile (10 µg/cm²). **p<0.05 versus Con. n = 3/group.

FIGURE 2. Chrysotile induces ER stress in bone marrow-derived macrophages. (A) Macrophages were exposed to chrysotile (10 µg/cm²), Tg (100 nM) or tunicamycin (TM, 5 µg/ml) for 24h, and then assayed for XBP1 splicing using conventional RT-PCR plus acrylamide gel electrophoresis. Data from experiments conducted as described in A are shown: (B) immunoblot analysis for BiP protein and β-actin, (C) densitometric analysis of BiP protein expression corrected for β-actin (*p<0.05 versus Con). (D,E,F) Quantitative RT-PCR data showing relative BiP mRNA, relative CHOP mRNA, and relative Tgfβ1 mRNA corrected β-actin. *p<0.05 versus Con, **p<0.05 versus other groups. N = 6-10/group.

FIGURE 3. Chrysotile increases cytosolic Ca²⁺ levels in macrophages. (A) Cells loaded with Fluo-3 AM (2.5 µM, 1h, 37 °C) and then exposed to chrysotile (10 µg/cm²) for evaluation by confocal microscopy. Cells loaded with Fura-2 AM (1µM, 30min, 37 °C), suspended in HBSS containing 1.3 mM Ca²⁺, and exposed to chrysotile (10 µg/well) followed by (B) 5 µM ionomycin or (C) 2 mM EDTA. Values are mean±sem (n=3/group). (D) Cells loaded with Fura-2 AM as above stated, suspended in Ca²⁺ free HBSS, exposed to chrysotile (10 µg/cm²) followed by 2 mM CaCl₂. (E) Cells loaded with Fura-2 AM (1 µM, 30min, 37 °C) and Ca²⁺ chelator BAPTA-AM (1 µM, 30min, 37 °C) 30 min before chrysotile exposure. The proton ionophore FCCP (5 µM) was used as a positive control. n=6/group. Cells exposed to BAPTA-AM (5 µM, 24h) and assayed for (F) spliced XBP1, by conventional RT-PCR plus gel electrophoresis as well as (G) BiP protein expression by immunoblot analysis and (H) BiP mRNA expression by quantitative RT-PCR. Thapsigargin (Tg) 100 nM for 24h as positive control. *p<0.05 versus control (n= 6/group).

FIGURE 4. Anisomycin treatment reduces cytosolic Ca²⁺ levels and modulates chrysotile-induced ER stress. Cells were loaded with Fura-2 AM (1 µM, 30min, 37 °C) and suspended in either (A) HBSS containing 1.3 mM Ca²⁺ HBSS or (B) Ca²⁺ free HBSS. Cells were incubated with 200 µM anisomycin for 60 min before exposure to chrysotile (10 µg/cm²). Values are mean±sem (n=6/group). (C) Macrophages were pretreated with 0.2 µM anisomycin for 60 min before exposure to chrysotile (10 µg/cm²) for 24h. BiP protein in lysates was determined by immunoblot analysis. Data from experiments conducted as described in C are shown: (D) densitometric analysis of BiP protein corrected for β-actin (mean±sem, 3 experiments), (E) BiP mRNA corrected for HPRT mRNA assayed by quantitative RT-PCR (*p<0.05 versus control, mean±sem, 3 experiments), and (F) XBP1 splicing evaluated by conventional RT-PCR followed by acrylamide gel electrophoresis.

FIGURE 5. Chrysotile increases ionomycin-releasable calcium stores and alters activation of store-operated Ca²⁺ channels in macrophages. (A) Cells were loaded with Fura-2 AM (1 µM, 30min, 37 °C), suspended in Ca²⁺ free HBSS, and exposed to chrysotile (10 µg/well) or Tg (100 nM) or TM (5 µg/ml)
Chrysotile induces ER stress in macrophages

followed by (B) 2 mM CaCl2. Values are mean±sem (n=6/group). (C) Cells were exposed to chrysotile (10 µg/well) or Tg (100 nM) or TM (5 µg/ml) for 24h and then assayed for Ca2+ release by ionomycin (5 µM). (D) Increments in cytosolic Ca2+ were determined by the maximum change in F340/380 observed in the first 2 min after ionomycin. *p<0.05 versus control (n= 6/group) (E) Cells were exposed to chrysotile, Tg, or TM for 24h as above, and then assayed for changes in cytosolic Ca2+ after addition of 2 mM CaCl2. (F) Increments in cytosolic Ca2+ after CaCl2 addition were determined as described in D. * or **p<0.05 versus control (n= 6/group) (E) Macrophages were transfected with either scrambled or IP3R-1 siRNA for 48h and assayed for (G) IP3R-1 mRNA by quantitative RT-PCR (*p<0.05 versus scramble) and (H) cytosolic Ca2+ levels after chrysotile exposure. (n = 6/group).

FIGURE 6. ER stress is present in macrophages obtained from fibrotic lungs. C57BL/6J mice were given either TiO2 (125 µg) or chrysotile (125 µg) intratracheally. BAL and lung tissues obtained 21d after exposure. (A) Lung sections from mice exposed to TiO2 or chrysotile were assayed by Masson’s Trichrome staining for detection of collagen deposition. (B) Hydroxyproline content of mouse lung exposed to TiO2 or chrysotile for 21d. *p<0.05 versus TiO2 (n= 6/group). (C) Differential counts for BAL cells after Giemsa-Wright staining (n = 6/group). (D) BAL cell lysates assayed by BiP protein immunoblotting. (E) Densitometric analysis of BiP protein expression corrected for β-actin *p<0.05 versus Con (n=4/group). (F) Total RNA from whole lung or from BAL cells was evaluated by quantitative RT-PCR for BiP mRNA normalized to β-actin mRNA. *p<0.05 versus other groups (n = 3/group). (G) BAL cell lysates assayed by protein immunoblotting for caspase-3 and β-actin. (H) Densitometric analysis of caspase-3 protein corrected for β-actin (n=4/group, p=0.06). (I) BAL fluid assayed for Tgf-β1 by ELISA. Values normalized to BAL protein, n=4-5/group.

FIGURE 7. Alveolar macrophages obtained from asbestosis patients show indicators of ER stress. (A) Alveolar macrophage lysates used for assay of BiP and ATF6 protein expression by immunoblotting. (B-C) Densitometric analysis of BiP and ATF6 protein expression normalized to β-actin in normal subjects and asbestosis patients. *p<0.05 versus normal subjects (n= 3-4/group). (D,E,F) Quantitative RT-PCR data showing mRNA of different ER stress genes corrected for HPRT mRNA. *p<0.05 versus normal subjects (n = 3/group).

The abbreviations used are: AEC, alveolar epithelial cells; ATF6, activating transcription factor 6; BAL, bronchoalveolar lavage; BiP, immunoglobulin binding protein; CHOP, DNA-damage inducible protein; Chry, chrysotile; ER, endoplasmic reticulum; ERO1-α, endoplasmic oxidase 1-α; Erp72, endoplasmic-resident protein 72; GRP78, glucose-regulated protein 78; GRP94, glucose-regulated protein 94; FCCP, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; IP3R-1, inositol 1,4,5-triphosphate receptor; IRE1, inositol-requiring enzyme 1; P58IPK, protein kinase inhibitor 58; PERK, PKR-like ER kinase; RT-PCR, Reverse transcription polymerase chain reaction; SP, surfactant protein; TGF-β1, transforming growth factor beta-1; TiO2, titanium dioxide; Tg, thapsigargin; TM, tunicamycin, UPR, unfolded protein response; XBP-1, X-box binding protein 1.
Chrysotile induces ER stress in macrophages

Figure 1

A

<table>
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<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
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</tr>
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<td>263 bp</td>
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Unspliced XBP1
Spliced XBP1

B

Chrysotile (h)

0  24  48

BiP
ATF6 α
β-actin

C

Relative BIP protein

Con  Chry  Tg

D

Relative BIP mRNA

Con  3  6  24  48  Tg  24h

E

Relative GRP94 mRNA

Con  Chrysotile

F

Relative ERP72 mRNA

Con  Chrysotile

G

Relative P58IPK mRNA

Con  Chrysotile
Chrysotile induces ER stress in macrophages

Figure 3

A

B

C

D

E

F

G

H
Chrysotile induces ER stress in macrophages

Figure 4

A

Cytosolic Ca²⁺ (340/380 Ratio) vs. Time (min)

Control
Chrysotile
Anisomycin (200 μM)
Anisomycin+Chrysotile

B

Cytosolic Ca²⁺ (340/380 Ratio) vs. Time (min)

Ca²⁺ Free HBSS

C

BiP
β-actin

Anisomycin
Chrysotile

D

Relative BiP protein

Chrysotile - + - + Anisomycin +

E

Relative BiP mRNA

Chrysotile - + - + Anisomycin +

F

Anisomycin
Chrysotile

269 bp
263 bp

Unspliced XBP1
Spliced XBP1
Chrysotile induces ER stress in macrophages

Figure 5

A  Ca²⁺ Free HBSS
   - Control  - Tg  - Chrysotile  - TM
   - Chrysotile, Tg or TM
   
   Cytosolic Ca²⁺ (340/380 Ratio)
   Time (min)
   0  10  20  30  40

B  Ca²⁺ Free HBSS
   - Control  - Tg  - Chrysotile  - TM
   - 2 mM CaCl₂
   
   Cytosolic Ca²⁺ (340/380 Ratio)
   Time (min)
   0  50  100  150

C  Ca²⁺ Free HBSS
   - Control  - Tg  - Chrysotile  - TM
   - Ionomycin
   
   Cytosolic Ca²⁺ (340/380 Ratio)
   Time (min)
   0  2  4  6  8  10

D  After Ionomycin

   Increment in Cytosolic Ca²⁺
   (Fₘₐₓ - Fₘᵢₙ)
   Con  Chry  Tg  TM

E  Ca²⁺ Free HBSS
   - Control  - Tg  - Chrysotile  - TM
   - 2 mM CaCl₂
   
   Cytosolic Ca²⁺ (340/380 Ratio)
   Time (min)
   0  2  4  6  8  10

F  After CaCl₂

   Increment in Cytosolic Ca²⁺
   (Fₘₐₓ - Fₘᵢₙ)
   Con  Chry  Tg  TM

G  si-Scr  - si-iP3R-1
   si-Scr + Chrysotile  - si-iP3R-1 + Chrysotile

H  Ca²⁺ Free HBSS
   - Chrysotile
   
   Cytosolic Ca²⁺ (340/380 Ratio)
   Time (min)
   0  15  30  45
Figure 6

**A**

TiO₂  
Chrysotile

**B**

Hydroxyproline (μg/g)  
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**C**

BAL Cells (%)  
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**D**

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<tr>
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<tr>
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<tr>
<td>β-actin</td>
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**E**

Relative BIP protein  
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**F**

Relative BIP mRNA  
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<td>Alveolar Macrophages</td>
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**G**

Caspase-3

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**H**

Relative Caspase 3 protein  
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**I**

BAL TGF-β1 (pg/mg)  
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Chrysotile induces ER stress in macrophages

Figure 7

A

B

C

D

E

F

Normalization

Asbestosis

Asbestosis

Subjects

Subjects

Subjects

Subjects

Relative BIP protein

Relative ATF6 protein

Relative BIP mRNA

Relative ERO1α mRNA

Relative CHOP mRNA

Normal

Asbestosis

Normal

Asbestosis

Normal

Asbestosis

Normal

Asbestosis

Normal

Asbestosis

Normal

Asbestosis

*
Asbestos-induced Disruption of Calcium Homeostasis Induces Endoplasmic Reticulum Stress in Macrophages
Alan J. Ryan, Jennifer L. Larson-Casey, Chao He, Shubha Murthy and A. Brent Carter

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