CHRONIC ARSENITE EXPOSURE IN LUNG EPITHELIUM MODULATES ENDOCYTOSIS

by

Anoop Singh Hunjan

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Walter T. Klimecki, DVM, Ph.D.
Associate Professor of Pharmacology and Toxicology

November 14, 2017
Date
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AWKNOWELEDGEMENTS

I would like to thank my advisor, Dr. Walter Klimecki of the department of Pharmacology and Toxicology at the University of Arizona, for all his support and mentorship throughout the past four years while working in his laboratory as both an undergraduate and graduate student. I would also like to thank my fellow lab members Eva Amouzougan and Scott Malm for all their support and guidance during my time in the Klimecki Laboratory.
ABSTRACT

Arsenic exposure in humans has been implicated in the development of a myriad of non-cancerous and cancerous diseases. A reductionist approach to understanding this unusual phenomenon would suggest that arsenic-induced perturbation of a small number of fundamental biological processes could manifest as this diverse array of disease endpoints. Endocytosis is a fundamental cellular process involved in the internalization and transport of various extracellular molecules and membranous components. BEAS-2B, a human bronchial epithelial cell line, was used to characterize the effects of chronic arsenite exposure on endocytosis. Fluorophore-labeled bovine albumin, human transferrin, and human low-density lipoprotein (LDL) were the substrates utilized to measure endocytosis in BEAS-2B cells. The uptake of albumin in unexposed BEAS-2B cells is both dose-dependent and temperature sensitive. Chronic arsenite exposure in BEAS-2B cells increased the uptake of albumin by 3.4-fold after 8 hours of uptake relative to unexposed BEAS-2B cells. Pharmacological studies utilizing endocytosis inhibitors suggested that the uptake of albumin in both unexposed and arsenite-exposed BEAS-2B cells occurs through a combination of receptor-mediated endocytosis and macropinocytosis. Chronic arsenite exposure in BEAS-2B cells also increased the endocytic uptake of transferrin by 2.9-fold at 30 minutes and LDL by 1.3-fold at 2 hours relative to unexposed BEAS-2B cells. Together, the data suggests that chronic arsenite exposure can increase the rate of endocytosis. This novel finding could add mechanistic insight to the conundrum of arsenic-associated human diseases.
Arsenic is a naturally occurring heavy metal, which is commonly found either in inorganic or organic states throughout the earth’s crust (1). Individuals are predominately exposed to arsenic through the environment, mining, and industrial manufacturing processes (1,2). Examples of environmental arsenic exposure include drinking contaminated water, inhalation, and eating contaminated agricultural produce (2). Exposure to arsenic has adverse health consequences, which can be both non-cancerous and cancerous, and can affect an array of organ systems. Examples of increased risk for non-cancerous diseases due to arsenic exposure include peripheral neuropathies, hypertension, atherosclerosis, proteinuria, and chronic obstructive pulmonary disease (3). Arsenic is formally classified by the International Agency for Research on Cancer (IARC) as an IARC group 1 carcinogen (4). Additionally, arsenic has been implicated in the development of a variety of cancers, for example, cancers of the liver, lung, and bone marrow (3,4).

We have observed cells cultured in fetal bovine serum (FBS) that were chronically exposed to arsenic had more bovine serum albumin (BSA) present by western blot analysis relative to unexposed FBS cells. These preliminary observations of increased albumin uptake in cells chronically exposed to arsenic were interesting to us initially for two main reasons: first potentially reflecting fundamental changes in cellular biology (e.g. endocytosis processes, changes in receptor expression, receptor recycling, etc.) and second, the potential for modulating the uptake of drugs that are conjugated or associated to albumin. The study of arsenic exposure and its consequences for endocytosis processes together may have significant clinical and public health implications.

Endocytosis is a complex cellular process that is involved in the internalization and transport of various extracellular molecules and membranous components (5). In addition to this, endocytosis is involved in the regulation of receptor-based cellular signaling (6). The process of endocytosis can generally be broken down into two forms; clathrin-mediated endocytosis (CME) and clathrin independent endocytosis (CIE) (7). Examples of CIE include caveolae mediated endocytosis and pinocytosis (7). Perturbations in endocytosis and related processes have been implicated in diseases including atherosclerosis, proteinuria, cancer, and cystic fibrosis (8, 9, 10, 11, 12). It is plausible that arsenic exposure can modulate endocytosis and potentially enhance disease development.

Epidemiological studies, for example, have demonstrated that individuals exposed to arsenic manifest symptoms of cystic fibrosis patients without having mutations in their cystic fibrosis transmembrane conductance regulator (CFTR) genes (13). Furthermore, a study by Bomberger et al. has already demonstrated that arsenic exposure in human airway epithelial cells increases the ubiquitylation and lysosomal degradation of the CFTR chloride channel protein (14). The endocytic trafficking of the CFTR protein is one of the mechanisms involved in the cystic fibrosis disease (12). The effects of arsenic exposure on the uptake of CFTR protein may similarly contribute to chronic lung infections as in cystic fibrosis patients (13, 15). Together, there is preliminary evidence suggesting that arsenic exposure may be able to modulate the endocytic uptake of key membranous components.

The main objective of this thesis project is to establish a model for the study of the effects of chronic arsenic exposure on endocytosis. We accomplish this by primarily utilizing fluorescently-tagged model endocytic substrates to determine endocytosis activity and the
mode(s) of endocytosis that were occurring. A chronically arsenite-exposed in vitro model was generated in BEAS-2B; a human bronchial epithelial cell line.
MATERIALS AND METHODS

Unexposed BEAS-2B Cell Culture
BEAS-2B (ATCC, Manassas, VA), an adenovirus 12-SV40 transformed normal human bronchial epithelium (16), was cultured in BEGM (BEBM supplemented with BEGM BulletKit) (Lonza, Walkersville, MD). 250,000 BEAS-2B cells were seeded into T75 cm² culture flasks and fed every 48 hours by aspirating the pre-existing growth medium and replacing it with fresh growth medium. The cells were subcultured when the culture reached 70-75% confluence. The BEAS-2B cells were washed with 1X DPBS (Corning, Corning, NY) and 0.25% trypsin/2.21 mM EDTA (Thermo Fisher Scientific, Carlsbad, CA) was added to the tissue culture flask at 37 °C for 4-5 minutes to detach the cells, and defined trypsin inhibitor (DTI) (Thermo Fisher Scientific, Carlsbad, CA) was subsequently added to inhibit the enzyme. Cells were then counted by using a Scepter 2.0 automated cell counter (MilliporeSigma, St. Louis, MO), and either seeded back and or used for experiments.

Arsenite-Exposed BEAS-2B Cell Culture
BEAS-2B cells were exposed to 1 μM sodium arsenite (MilliporeSigma) for at least 40 weeks. The sodium arsenite concentration was prepared fresh in BEGM during passing or feeding. Arsenite-exposed cells were maintained on the same schedule as the unexposed BEAS-2B cells for subculturing and experiments.

Dose-Dependent Albumin Uptake by Flow Cytometry
Unexposed BEAS-2B cells were seeded at 400,000 cells per well in 6-well plates. After 24 hours, the cells were administered either 0, 25, 50, 75, or 100 μg/mL Alexa Flour 488 BSA (AF-488 BSA) conjugate (Thermo Fisher Scientific) for 1 hour.

Temperature Sensitive Albumin Uptake by Flow Cytometry
Unexposed BEAS-2B cells were seeded at 200,000 cells per well in 6-well plates. After 48 hours, a plate was transitioned from 37 °C to 4 °C for 1 hour. After this initial transition period, the cells were administered 50 μg/mL AF-488 BSA for 0, 15, 30, 45, and 60 minutes. The 37 °C and 4 °C plates were maintained at their respective conditions throughout the experiment. Additionally, the reagents were also kept at either 37 °C or 4 °C to help keep the temperature of the respective systems relatively constant.

Albumin Uptake by Flow Cytometry
Unexposed and arsenite-exposed BEAS-2B cells were seeded at 400,000 cells per well in 6-well plates. After 24 hours, the cells were administered 50 μg/mL AF-488 BSA for 0, 1, 2, 4, and 8 hours.

LDL and Transferrin Uptake by Flow Cytometry
Unexposed and arsenite-exposed BEAS-2B cells were seeded at 200,000 cells per well in 6-well plates. After 48 hours, the cells were administered with either 7.5 μg/mL BODIPY FL human low-density lipoprotein (BODIPY-FL LDL) complex (Thermo Fisher Scientific) for 2 hours or 50 μg/mL Alexa Flour 488 Transferrin (AF-488 transferrin) conjugate (Thermo Fisher Scientific) for 30 minutes.
**Endocytosis Inhibition by Flow Cytometry**

Unexposed and arsenite-exposed BEAS-2B cells were seeded at 200,000 cells per well in 6-well plates. To investigate the mode of endocytosis responsible for the uptake of albumin, the following pharmacological inhibitors were tested: Cytochalasin D (MilliporeSigma), Latrunculin B (MilliporeSigma), and Pitstop 2 (ABCAM, Cambridge, UK). After 48 hours, the cells were pre-treated for an hour with a concentration of 0, 5, or 20 μM of the respective inhibitors. DMSO (MilliporeSigma) was used as a vehicle for Cytochalasin D and Pitstop 2 treatments, and ethanol (MilliporeSigma) was used as a vehicle for Latrunculin B treatments. The cells were then administered 50 μg/mL AF-488 BSA for 2 hours and the concentration of the respective pharmacological inhibitors were maintained throughout the duration of the experiments.

**Endosomal Acidification Inhibition by Flow Cytometry**

Unexposed and arsenite-exposed BEAS-2B cells were seeded at 100,000 cells per well in 6-well plates. After 72 hours, the cells were pre-treated with Bafilomycin A1 (Santa Cruz Biotechnologies, Santa Cruz, CA) for 2.5 hours at a concentration of 0 or 10 nM. DMSO was utilized as a vehicle for Bafilomycin A1 treatments. The cells were then administered 50 μg/mL AF-488 BSA for 30 minutes and the concentration of Bafilomycin A1 was maintained throughout the duration of the experiment.

**Proteasome Inhibition by Flow Cytometry**

Unexposed and arsenite-exposed BEAS-2B cells were seeded at 200,000 cells per well in 6-well plates. After 48 hours, the cells were pre-treated for 2 hours with MG132 (MilliporeSigma) at a concentration of 0 or 10 μM. The cells were then administered 50 μg/mL AF-488 BSA for 2 hours and the concentration of MG132 was maintained throughout the duration of experiment.

**Endosomal Doxorubicin Delivery by Flow Cytometry**

Unexposed and arsenite-exposed BEAS-2B cells were seeded at 200,000 cells per well in 6-well plates. After 48 hours, 10 μM doxorubicin (MilliporeSigma) was pre-incubated with either 100 μg/mL BSA (MilliporeSigma) or 100 μg/mL Alexa Flour 680 BSA (AF-680 BSA) conjugate (Thermo Fisher Scientific) in growth medium for 1 hour at 37 °C. After 1 hour, the sample was pushed through a 5K molecular weight cut-off (MWCO) spin-filter (Corning) at 16,000 RPM for 15 minutes at room temperature (RT). The retentate was removed and resuspended in growth medium, and the solution was then administered to the cells for 2 hours.

**Collection of Samples for Flow Cytometry**

The cells were washed using 1X DPBS, incubated with 0.25% trypsin/2.21 mM EDTA, and then DTI was used to inhibit the enzyme. The samples were then immediately spun down at 1000 RPM for 5 minutes at RT and fixed by aspirating the supernatant and re-suspending the cell pellet in 4% paraformaldehyde (Ted Pella Inc., Redding, CA) for 20 minutes at RT. After fixation, the cells were spun-down once more at 1000 RPM for 5 minutes at RT and then re-
suspended in 1X DPBS. The samples were stored in the dark and at 4 °C until analysis by flow cytometry.

*Flow Cytometry Analysis*

Analysis of flow cytometry experiments were performed using a BD LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and BD FACSDIVA software (Becton Dickinson). An excitation wavelength of 488 nm was used for the different AF-488 BSA, AF-488 transferrin, and BODIPY-FL LDL experiments. Additionally, an excitation wavelength of 488 nm was used for doxorubicin and 700 nm for AF-680 BSA for the endosomal doxorubicin delivery experiments. The total event count for each of the samples ran on the flow cytometer was 10,000. Data analysis was performed using Flowing 2 Software (Developed by Perttu Terho, Turku Centre for Biotechnology). Flow cytometry sample acquisition was at the courtesy of the Flow Cytometry Core Facility at Arizona Research Laboratories.

*Localization of Transferrin by Confocal Microscopy*

Unexposed and arsenite-exposed BEAS-2B cells were seeded at 400,000 cells per well in 6-well plates that contained #1.5H glass coverslips (Bioscience Tools, San Diego, CA) that were previously sterilized and coated with a collagen and fibronectin solution (MilliporeSigma). After 24 hours, the growth medium was removed, and the cells were administered 50 μg/mL AF-488 transferrin for 0, 15, and 30 minutes in fresh growth medium. Cells were then immediately washed with 1X DPBS and fixed using 4% paraformaldehyde at RT for 20 minutes. Post-fixation, the cells were labeled with 5 unit/mL DyLight 554 Phalloidin (Thermo Fisher Scientific) for 1 hour at RT and then next labeled with 300 nM DAPI (Thermo Fisher Scientific) at RT for 7 minutes. The coverslips were washed with DPBS first and then washed a second time using Milli-Q water, and then the coverslips were mounted onto glass slides using SlowFade Diamond Antifade Mountant (Thermo Fisher Scientific).

*Confocal Microscopy Analysis*

Images were acquired using a Leica SP5-II spectral confocal microscope (Leica, Wetzlar, Germany) using a 63x/1.4 NA apochromatic oil objective. Additionally, excitation wavelengths of 405, 488, and 543 nm were used to excite and detect the respective fluorophores. 3 random Z-stacks containing 20 slices were taken per sample. The apical and basolateral side of the cells were identified by using the phalloidin stain. Maximum intensity projection of certain fields of view were generated to better understand the localization of the endocytic substrate. The acquisition and analysis of images were performed using Leica LAS-X software. The acquired images were exported as TIF files. Additional image analysis was performed using the ImageJ software (17). The image acquisition and analysis were at the courtesy of the Imaging Shared Resource at the Arizona Cancer Center.

*Statistical Analysis*

Statistical analysis of data was performed using GraphPad Prism version 7 for Windows (GraphPad, La Jolla, CA). Unpaired t-tests with Welch’s correction were used to compare the mean differences of data that contained two comparison groups at a statistical significance threshold of P<0.05.
RESULTS

Figure 1

To determine an effective concentration of AF-488 BSA to utilize for this project; an AF-488 BSA dose response was performed. Unexposed BEAS-2B cells were administered varying concentrations of AF-488 BSA ranging from 0-100 μg/mL for 1 hour. The AF-488 mean fluorescent intensity (MFI) increases as the concentration of AF-488 BSA increases. No saturability for the uptake of AF-488 BSA was detected for the concentrations tested at this 1-hour timepoint. Based upon these results, a concentration of 50 μg/mL AF-488 BSA was selected to assess the uptake of AF-488 BSA in this project.

Dose-Dependent Uptake of AF-488 BSA in Unexposed BEAS-2B Cells

A summary figure of 2 biological replicates (error bars represent standard error of the mean [SEM]), and each of the biological replicates contained 2 technical replicates per group.
Figure 2

To verify that the uptake of AF-488 BSA required physiologic conditions, the temperature dependence of BSA uptake was evaluated. The uptake of AF-488 BSA in unexposed BEAS-2B cells was compared at 37 °C and 4 °C. The cells were administered a concentration of 50 μg/mL AF-488 BSA for 0, 15, 30, 45, and 60 minutes. The uptake of AF-488 BSA appears to require active biological processes, as we observed the uptake of AF-488 BSA to be reduced at 4° C relative to 37° C.

**Temperature Sensitive Uptake of AF-488 BSA in Unexposed BEAS-2B Cells**

![Temperature Sensitive Uptake of AF-488 BSA](image)

A summary figure of 4 biological replicates (error bars represent SEM, unpaired t-tests with Welch’s correction, *P<0.0332, **P <.0.0021), and each of the biological replicates contained 2 technical replicates per group.
To better understand the effects of chronic arsenite exposure on the uptake of AF-488 BSA, the uptake of AF-488 BSA in unexposed and arsenite-exposed BEAS-2B cells was compared in a 0 to 8-hour time course. Cells were administered a concentration of 50 μg/mL AF-488 BSA throughout the time course. The uptake of AF-488 BSA is greater in the arsenite-exposed BEAS-2B cells across all the measurements. At 8 hours there is a 3.4-fold increase in AF-488 MFI for BSA uptake in arsenite-exposed BEAS-2B cells relative to unexposed BEAS-2B cells.

AF-488 BSA Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells

A summary figure of 3 biological replicates (error bars represent SEM, unpaired t-tests with Welch’s correction, *P<0.0332, **P <.0.0021), and the first 2 biological replicates contained 1 technical replicate per group and the last biological replicate contained 2 technical replicates per group.
**Figure 4**

To determine if chronic arsenite exposure also increases the uptake of other endocytic substrates in BEAS-2B cells; the uptake of both AF-488 transferrin and BODIPY-FL LDL was assessed. The uptake of AF-488 transferrin was evaluated between unexposed and arsenite-exposed BEAS-2B cells at the 30-minute timepoint using a concentration of 50 μg/mL AF-488 transferrin. There is a 2.9-fold increase in AF-488 MFI for transferrin uptake in arsenite-exposed BEAS-2B cells relative to unexposed BEAS-2B cells (Fig. 4A). The uptake of BODIPY-FL LDL was evaluated between unexposed and arsenite-exposed BEAS-2B cells at the 2-hour timepoint using a concentration of 7.5 μg/mL BODIPY-FL LDL. There is a 1.3-fold increase in AF-488 MFI for LDL uptake in arsenite-exposed cells relative to unexposed BEAS-2B cells (Fig. 4B).

**A. AF-488 Transferrin Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells**

![Graph showing AF-488 transferrin uptake in unexposed and arsenite-exposed BEAS-2B cells](image)

A summary figure of 2 biological replicates for AF-488 transferrin experiments (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.
B. BODIPY-FL LDL Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells

A summary figure of 4 biological replicates for BODIPY-FL LDL experiments (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.
Figure 5

The mode of albumin uptake was explored in unexposed and arsenite-exposed BEAS-2B cells by utilizing different endocytosis inhibitors. Pitstop 2, an inhibitor of CME, was tested to determine if CME was involved in the uptake of albumin (18). Both unexposed and arsenite-exposed BEAS-2B cells were treated for 1 hour with Pitstop 2 with concentrations ranging from 0, 5, 10, and 20 μM prior to administering the cells with a concentration of 50 μg/mL AF-488 BSA for 2 hours (3-hour total treatment time with inhibitor). Unexposed and arsenite-exposed BEAS-2B cells were normalized to their respective AF-488 BSA uptake at 2 hours for 0 μM Pitstop 2 inhibitor (Fig. 5A). Cytochalasin D and Latrunculin B are both inhibitors of actin polymerization, and are commonly used to inhibit macropinocytosis (19, 20). The Cytochalasin D (Fig. 5B) and Latrunculin B (Fig. 5C) experiments were performed likewise to the Pitstop 2 inhibitor experiments. Overall, the curves for unexposed and arsenite-exposed BEAS-2B cells overlapped closely suggesting that the mode of AF-488 BSA uptake may be occurring through similar CME and macropinocytosis like pathways.

A. Effects of Pitstop 2 on AF-488 BSA Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells

![Graph showing the effects of Pitstop 2 on AF-488 BSA uptake](image)

A summary figure of 3 biological replicates for the AF-488 BSA Pitstop 2 inhibitor experiments (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.
B. Effects of Cytochalasin D on AF-488 BSA Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells

A summary figure of 3 biological replicates for the AF-488 BSA Cytochalasin D inhibitor experiments (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.

C. Effects of Latrunculin B on AF-488 BSA Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells

A summary figure of 3 biological replicates for the AF-488 BSA Latrunculin B inhibitor experiments (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.
Figure 6
The effects of endosomal acidification on albumin uptake was investigated in unexposed and arsenite-exposed BEAS-2B cells. The late endosome or lysosome is one of the last steps in endocytosis and it is involved in the degradation of certain receptor-ligand complexes (21). Bafilomycin A1, a specific inhibitor of the vacuolar H\(^+\) ATPase pump, prevents proper acidification required for organelle functionality (22). Both unexposed and arsenite-exposed BEAS-2B cells were pre-treated for 2.5 hours with a concentration of either 0 or 10 nM of Bafilomycin A1. After pre-treatment for 2.5 hours, the cells were administered a concentration of 50 μg/mL AF-488 BSA for 30 minutes (3-hour total treatment time with inhibitor). Unexposed and arsenite-exposed BEAS-2B cells were normalized to their respective AF-488 BSA uptake at 30 minutes for 0 nM Bafilomycin A1 inhibitor. Overall, both unexposed and arsenite-exposed BEAS-2B cells have reduced AF-488 BSA uptake in the presence of Bafilomycin A1.

**Effects of Bafilomycin A1 on AF-488 BSA Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells**

A summary figure of 3 biological replicates (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.
The effects of proteasome inhibition on the uptake of albumin in unexposed and arsenite-exposed BEAS-2B cells was explored. Inhibition of the proteasome in the literature has been demonstrated to inhibit the uptake of certain endocytic substrates. MG132, a reversible proteasome inhibitor, has been shown in a study by Yu and Malek to inhibit receptor mediated endocytosis of interleukin-2 (23, 24). Both unexposed and arsenite-exposed BEAS-2B cells were pre-treated with a concentration of either 0 or 10 μM MG132 for 2 hours, and then were subsequently administered a concentration of 50 μg/mL AF-488 BSA for 2 hours (4-hour total treatment time with inhibitor). Unexposed and arsenite-exposed BEAS-2B cells were normalized to their respective AF-488 BSA uptake at 2 hours for 0 μM MG132 inhibitor. Overall, arsenite-exposed BEAS-2B cells have reduced AF-488 BSA uptake in the presence of MG132 relative to unexposed BEAS-2B cells.

**Effects of MG132 on AF-488 BSA Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells**

A summary figure of 3 biological replicates (error bars represent SEM, unpaired t-tests with Welch’s correction, **P < 0.0021), and each of the biological replicates contained 2 technical replicates per group.
Figure 8

The effects of endosomal acidification inhibition were also investigated on the uptake of AF-488 transferrin. Both unexposed and arsenite-exposed BEAS-2B cells were pre-treated for 2.5 hours with either 0 or 10 nM Bafilomycin A1. After pre-treatment for 2.5 hours, the cells were administered a concentration of 50 μg/mL AF-488 transferrin for 30 minutes (3-hour total treatment time of inhibitor). Unexposed and arsenite-exposed BEAS-2B cells were normalized to their respective AF-488 transferrin uptake at 30 minutes for 0 nM Bafilomycin A1 inhibitor. Overall, arsenite-exposed BEAS-2B cells have reduced transferrin uptake in the presence of Bafilomycin A1 relative to unexposed BEAS-2B cells.

Effects of Bafilomycin A1 on AF-488 Transferrin Uptake in Unexposed and Arsenite-Exposed BEAS-2B Cells

Summary figure of 3 biological replicates (error bars represent SEM, unpaired t-tests with Welch’s correction, *P<0.0332), and each of the biological replicates contained 2 technical replicates per group.
Figure 9

To determine the cellular localization of AF-488 transferrin; unexposed and arsenite-exposed BEAS-2B cells were observed by confocal microscopy. Unexposed and arsenite-exposed BEAS-2B cells were administered a concentration of 50 μg/mL AF-488 transferrin for 0, 15, and 30 minutes. Post-fixation the cells were labeled with phalloidin and DAPI. Overall, arsenite-exposed BEAS-2B cells (Fig. 9B) have a greater cytosolic transferrin localization over the time course relative to unexposed BEAS-2B cells (Fig. 9A).

A. AF-488 Transferrin Localization in Unexposed BEAS-2B Cells by Confocal Microscopy

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>DAPI</th>
<th>AF-488 Transferrin</th>
<th>Phalloidin</th>
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A summary figure of 1 of 2 biological replicates for AF-488 transferrin localization experiments in unexposed BEAS-2B cells. Each of the images in this figure are maximum intensity projections of a z-stack containing 20 sections.
B. AF-488 Transferrin Localization in Arsenite-Exposed BEAS-2B Cells by Confocal Microscopy

A summary figure of 1 of 2 biological replicates for AF-488 transferrin localization experiments in arsenite-exposed BEAS-2B cells. Each of the images in this figure are maximum intensity projections of a z-stack containing 20 sections.
Figure 10

To investigate the effects of chronic arsenite exposure on albumin based drug delivery; the uptake of doxorubicin associated to albumin was explored in unexposed and arsenite-exposed BEAS-2B cells. Doxorubicin, an anti-cancer drug that inhibits DNA synthesis and disrupts topoisomerase-II-mediated DNA repair, was pre-incubated at 37 °C with either 100 μg/mL unlabeled or AF-680 BSA for 1 hour and then put through a spin-filter (25). The retentate containing albumin associated with doxorubicin was administered to unexposed and arsenite-exposed BEAS-2B cells for 2 hours. Approximately 71% of doxorubicin is found bound to proteins in total serum (26). In both cases, arsenite-exposed BEAS-2B cells have a greater uptake of doxorubicin associated to both unlabeled BSA (Fig. 10A) and AF-680 BSA (Fig. 10B) relative to unexposed BEAS-2B cells.

A. Doxorubicin Drug Delivery by Unlabeled BSA in Unexposed and Arsenite-Exposed BEAS-2B Cells

A summary figure of 2 biological replicates for the unlabeled BSA doxorubicin associated endosomal delivery experiments (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.
B. Doxorubicin Drug Delivery by AF-680 BSA in Unexposed and Arsenite-Exposed BEAS-2B Cells

A summary figure of 3 biological replicates for the AF-680 BSA doxorubicin associated endosomal delivery experiments (error bars represent SEM), and each of the biological replicates contained 2 technical replicates per group.
In this thesis project, the uptake of albumin was characterized in unexposed and chronically arsenite-exposed BEAS-2B cells by utilizing AF-488 BSA. Unexposed BEAS-2B cells exhibited dose-dependent uptake of AF-488 BSA; where the MFI of fluorescent albumin detected was concentration dependent at 1 hour (see Fig. 1). Additionally, we did not detect saturation of this albumin uptake system for the concentrations and timepoints tested. In a study by Choi et al., the authors could detect the saturability of albumin uptake in their opossum kidney epithelial model (27). Therefore, it may be possible to saturate the albumin uptake system in our model at higher concentrations than we tested. The uptake of AF 488-BSA in unexposed BEAS-2B cells was also temperature sensitive and showed reduced uptake when the system was at 4 °C (see Fig. 2). The reduced uptake of albumin at 4 °C has been demonstrated by Choi et al. and by Yumoto et al. in a rat derived lung alveolar epithelial model, and both are consistent with the temperature sensitivity of albumin uptake presently observed (27, 28). These findings suggest that the detection of AF-488 BSA at 4 °C may be due to cell surface binding in unexposed BEAS-2B cells.

The main objective of this study was to investigate chronic arsenite exposure as a modulator of endocytosis. This objective was first addressed by examining the uptake of AF-488 over an 8-hour time course. Throughout each timepoint BEAS-2B cells chronically exposed to arsenite always had a greater uptake of AF 488-BSA over unexposed BEAS-2B cells (see Fig. 3). This observation suggested that there may be fundamental changes in endocytic pathways occurring in BEAS-2B cells chronically exposed to arsenite. These initial findings of increased AF-488 BSA uptake in our chronically arsenite-exposed BEAS-2B cells were robust, and help corroborate the increased albumin uptake we have observed in our arsenite-exposed models by western blot analysis.

We next wanted to determine if the effect of arsenite on BSA endocytosis was unique to this substrate, or generalizable to multiple endocytotic substrates. To characterize this, we tested transferrin and LDL, which are both well-studied receptor mediated endocytic substrates. We observed that both transferrin and LDL had greater endocytic uptake in cells chronically exposed to arsenite (see Fig. 4). Collectively, this suggests that chronic arsenite exposure may be fundamentally altering the expression of receptor mediated endocytosis machinery, receptor expression, and or receptor recycling.

After these initial steps investigating the effects of chronic arsenite exposure on endocytosis, our attention shifted towards assessing the actual mode of endocytosis responsible for the uptake of albumin in this model. By utilizing multiple pharmacological inhibitors of molecular components involved in endocytosis, we aimed to better understand the mode of substrate uptake in our model. The uptake of AF 488-BSA was significantly reduced as the concentrations of Pitstop 2 administered was increased, and this suggested that the uptake of albumin was predominantly occurring through a CME process in both unexposed and arsenite-exposed BEAS-2B cells (see Fig. 5A). In addition to this, the involvement of macropinocytosis in the uptake of albumin was investigated by utilizing Cytochalasin D and Latrunculin B. Both Cytochalasin D and Latrunculin B exhibited similar behavior where the system reached a maximum level of inhibition, and started to plateau at approximately 60-80% to that of the respective no inhibitor controls for both unexposed and arsenite-exposed BEAS-2B cells (see Figs. 5B and 5C).
As the different modes of endocytosis employed in the uptake of AF-488 BSA were considered, the specificity of these respective endocytosis inhibitors also came into question. Further inquiry in the literature revealed that Pitstop 2, Cytochalasin D, and Latrunculin B are rather broad pharmacological inhibitors that can affect a variety of cellular processes. Dutta et al. demonstrated in their study that by utilizing a clathrin knockdown model that Pitstop 2 not only inhibits CME, but it can also perturb CIE processes (29). This makes it difficult to establish with certainty what the reduction of albumin uptake may be due to in our model when Pitstop 2 is administered. Additionally, a review by Kaksonen et al., highlights the importance of actin polymerization in nearly all modes of endocytosis, and not just simply macropinocytosis (30). Nonetheless, the uptake of albumin in our model may be occurring either through CME or CIE processes, and or potentially through a combination of both. The similarity in the extent of endocytosis inhibition between unexposed and arsenite-exposed BEAS-2B cells suggests that similar endocytotic processes are involved in both, and the activity of these processes may be modulated by arsenite exposure.

Additional experiments were performed to help better validate the modes of endocytosis responsible for albumin uptake. The focus shifted towards inhibiting endosomal acidification and determining if proper acidification was necessary in the uptake of albumin. We tested the inhibition of the vacuolar H⁺ ATPase pump by Bafilomycin A1, and saw that AF-488 BSA uptake was reduced in both unexposed and arsenite-exposed BEAS-2B cells (see Fig. 6). This finding was consistent with another study done by Yumoto et al., which demonstrated the inhibition of albumin uptake when cells were pre-treated with Bafilomycin A1 (31). Proper endosomal acidification is necessary for the dissociation of a ligand from its receptor within recycling endosomes (32). This measure orthogonally helps to verify that albumin uptake in our model is occurring through receptor mediated endocytosis (RME) or CME that requires proper receptor recycling. Furthermore, there appears to be no differential lysosomal degradation of AF-488 BSA suggesting that the effects of arsenite-exposure may be more upstream in the RME pathway that allows for differential accumulation of AF-488 BSA.

The requirements of the ubiquitin proteasome system for the endocytosis of albumin was also investigated by MG132. Treatment of cells with MG132 significantly reduced the uptake of AF-488 BSA in arsenite-exposed BEAS-2B cells relative to unexposed BEAS-2B cells (see Fig. 7). In both cases, this suggests that the ubiquitin proteasome system may be involved in the uptake AF-488 BSA. MG132 treatment has been demonstrated to deplete the free ubiquitin pool in a study by Patrick et al., which investigated the requirements of the ubiquitin pool for agonist induced endocytosis of glutamate receptors in a neuronal model (33). Additionally, ubiquitin modifications are commonly utilized as signals to facilitate the endocytosis of receptors and involved in endosomal sorting (34, 35). The results from this present experiment make it difficult to conclude at which step MG132 inhibition of the proteasome is inhibiting RME and or endosomal sorting. Altogether, the data suggests that proteasome functionality is required for the differential uptake of AF-488 BSA between unexposed and arsenite-exposed BEAS-2B cells. This also suggests that the differential AF-488 BSA levels between unexposed and arsenite-exposed BEAS-2B cells are not due to differential proteasomal degradation of AF-488 BSA.

As an additional measure, we verified the findings of AF-488 BSA in our model by also looking at the requirements for endosomal acidification for the uptake AF-488 transferrin. The transferrin receptor and ligand are a well-studied receptor-ligand system in which both the receptor and or ligand can be recycled back to the membrane (36). The inhibition of endosomal acidification by Bafilomycin A1 also diminished the uptake of AF-488 transferrin in both
unexposed and chronically arsenite-exposed BEAS-2B cells (see Fig. 8). These results are consistent with a study by Presley et al., which showed that Bafilomycin A1 treatment significantly reduces transferrin receptor recycling (37). Together, this data supports that AF-488 BSA endocytosis in our model is occurring through a similar RME pathway, which may require proper recycling of the receptor(s) back to the cell membrane. Lastly, we wanted to validate that the internalization of endocytic substrates was truly intracellular in our model. The localization of AF-488 transferrin was assessed over a brief 30-minute time course. By confocal microscopy there was greater intracellular localization at 30 minutes detected for AF-488 transferrin in arsenite-exposed BEAS-2B cells relative to unexposed BEAS-2B cells (see Figs. 9A and 9B). The AF-488 transferrin observed throughout multiple fields of view was largely perinuclear and within the boundaries of the phalloidin actin stain. Together, this establishes that the AF-488 transferrin that is being detected at these timepoints is predominantly within the cytosolic space of the cell. We also attempted to demonstrate the intracellular localization of the AF-488 BSA endocytic substrate in our model by confocal microscopy, but we were not able to achieve comparable levels of detection using similar timepoints and concentrations (data not shown).

As a part of the last aim for this project, the effects of chronic arsenite exposure on albumin associated drug delivery was assessed. We attempted to demonstrate this in our model by incubating unlabeled and fluorescently labeled BSA with doxorubicin, and then filtering it using specific MWCO filters. Both unlabeled and fluorescently labeled BSA variants were tested to account for the fluorescent properties of the labeled BSA molecule that may alter the binding of doxorubicin. The doxorubicin that was retained by BSA was associated with BSA through non-covalent protein interactions. In either case, BEAS-2B cells chronically exposed to arsenite showed greater fluorescence uptake of doxorubicin by BSA relative to unexposed BEAS-2B cells (see Figs. 10A and 10B). In another experiment, we observed that BEAS-2B cells chronically exposed to arsenite had a greater uptake of doxorubicin relative to unexposed BEAS-2B cells while independent of any sort of albumin associated drug delivery (data not shown). In conclusion, the uptake of free doxorubicin not associated to BSA and doxorubicin associated to BSA cannot be properly distinguished in this present experimental model. To answer this question more effectively, in the future, a fluorescent drug that is covalently linked to an albumin molecule can be utilized instead.

Overall, in this project we have demonstrated that chronic arsenite exposure increases the active process of endocytosis. BEAS-2B cells chronically exposed to arsenite had a greater uptake of albumin, LDL, and transferrin relative to unexposed BEAS-2B cells. Additionally, these preliminary findings suggest that the uptake of albumin in our model is occurring through similar pathways in unexposed and chronically arsenite-exposed BEAS-2B cells. It appears with the present pharmacological inhibitors tested the uptake of albumin is heterogeneous in this model, and may be occurring through both CME and CIE processes. In future studies, we could utilize additional pharmacological inhibitors with greater specificity and or transient inhibition of endocytic machinery to determine the mode of albumin endocytosis more precisely in both unexposed and arsenite-exposed BEAS-2B cells. Lastly, the findings from this work have significant implications for endocytic mechanisms that may underlie arsenic induced diseases. Arsenic exposed individuals, for example, may have a greater risk for the development of atherosclerosis due to increased LDL uptake in certain tissues. Likewise, the perturbations in endocytosis observed due to arsenic exposure may also translate to a higher risk for viral infection of cells that comprise the respiratory airways. Nonetheless, the findings from this project may serve as a future foundation to investigate the effects of arsenic exposure on the
endocytosis of other receptor-ligand complexes, expressional changes in endocytic machinery, and or if there is an involvement of dysregulated endocytic pathways that facilitate certain lung pathologies.
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