Ultrafine carbon nanoparticles activate inflammasome signaling and cell death in murine macrophages

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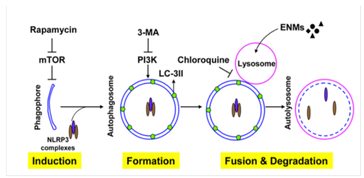
Abstract

Carbon black (CB) is the primary nanoparticulate component of air pollution from fossil fuel combustion. Prior research has demonstrated that CB induces elevated reactive oxygen species and cellular stress signaling upon cellular accumulation via receptor-mediated endocytosis. Recent published work has identified particulates of asbestos and some rare earth elements to have the capacity to activate pro-inflammatory inflammasome signaling from immune system cell types. This work examines the cellular impact of ultrafine carbon (carbon black, CB) nanoparticles, that range in size down to 30 nm, upon murine macrophages. CB nanoparticles were prepared via sonication in a buffered bovine serum albumin solution. The size analysis of the carbon black nanoparticles was performed using atomic force microscopy (AFM) and transmission electron microscopy (TEM) techniques. RAW246.7 macrophage cells were exposed to CB doses ranging from 50 - 200 ug/ml in complete media. Analysis of cell survival over time revealed elevated rates of significant nuclear degradation and cell lifting after 48 hours of exposure, and in a dose dependent pattern. Live cell imaging of cells exposed to nanoparticles revealed a visible uptake of nanoparticles with accumulation over time. To assess inflammasome signaling, both caspase-1 activation and IL-1b production were observed in whole cell lysates. Caspase-1 activation was measured as the appearance of the active (cleaved) form of the protease appearing in immunoblot analysis. Caspase-1 activity is responsible for proteolytic processing of pro-IL-1β to IL-1β subsequent to release from macrophages. Immunoblot analysis revealed significant activation of caspase-1 with 48-hour CB exposures at doses of 100-200 ug/ml. Similarly, levels of IL-1β were significantly induced by CB exposure, with maximal induction observed after a 48 hour exposure. As particulate accumulation in macrophages have been shown to disrupt the autophagic degradation pathway, macrophage cells were assessed for accumulation of LC3, a marker for autophagosome vesicles. Immunoblot analysis revealed a significant accumulation of LC3 in response to CB exposure and in response to chloroquine, which inhibits autophagosome/lysosome fusion. Further analysis of autophagy pathways via microscopy will be discussed. Taken together, these results support a model in which CB exposure activates the inflammasome and disrupts autophagy in macrophages.

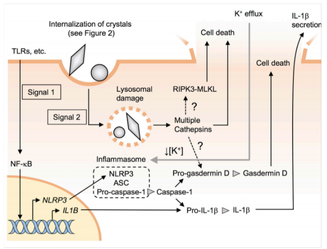
Introduction

Lysosomes are organelles that act as the terminal location in cytolytic pathways and are thus involved in cellular recycling and defense. Lysosomes complete these tasks through proton pumps to maintain low (4.6-5.0) pH, specialized proteases titled cathepsins, and fusogenic properties with autophagosomes[2]. When lysosomal membranes are damaged, cathepsins and protons can leak into the cell and, since lysosomal membrane permeabilization can occur with nanoparticulates (NPs), are thus an indication of cellular stress. Lysosomal-mediated autophagy is a starvation and damage induced mechanism that allows the cell to degrade its components and enact an immune response.[3] The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that acts as a signal for growth, development, cytoskeleton organization, and survival. Without mTOR presence, a set of autophagy proteins called *Atg* proteins elongate the phagophore during attachment to the target and convert the cytosolic protein LC3-I into LC3-II. After the closing of the autophagosome, lysosomes fuse and the contained items are degraded by lysosomal hydrolases.



Model 1: Overview of Autophagy[4]

Carbon black (CB) is a nanoparticle component of environmental pollution and is created through carbon combustion reactions. CB has been shown to induce inflammation, oxidative stress, aggravation of asthma, decreased cellular propagation, fragmented mitochondria, and increased reactive oxygen species (ROS) in human lung cells.[1] In addition to the aforementioned dangers, certain NPs such as rare earth oxides (REOs) have been shown to prevent the critical process of cellular homeostasis of autophagy.[4] Finally, the deregulation of autophagy thwarts a necessary cellular pro-survival mechanism and has been linked to cancer, neurodegenerative diseases such as Parkinson's disease, and lysosomal storage diseases (LSDs) such as Tay-Sachs syndrome.[5] These findings indicate the damaging cellular role of NPs as they relate to lack of cellular control, cancer, inflammation, and overall histological damage.



Model 2: Relationship between NPs, Inflammasome, and Apoptosis[6]

Results

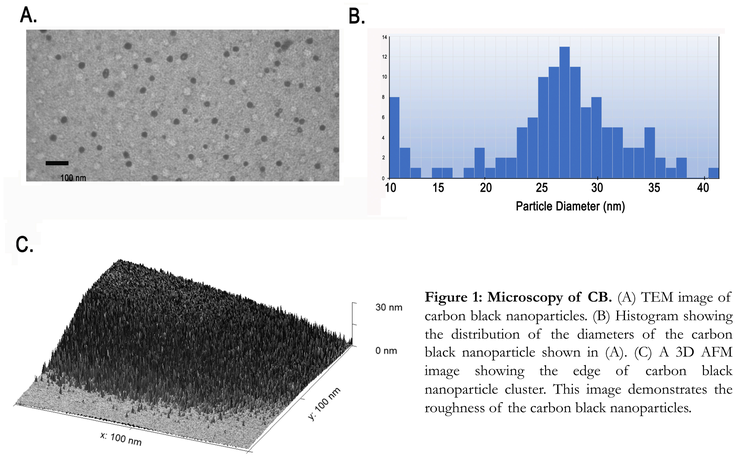


Figure 1: Microscopy of CB. (A) TEM image of carbon black nanoparticles. (B) Histogram showing the distribution of the diameters of the carbon black nanoparticle shown in (A). (C) A 3D AFM image showing the edge of carbon black nanoparticle cluster. This image demonstrates the roughness of the carbon black nanoparticles.

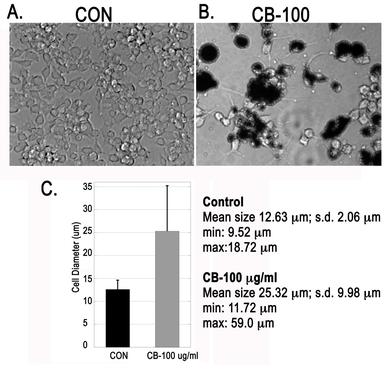


Figure 2: Chronic exposure to CB leads to accumulation of particles in the cells and an increase in cell diameter. (A/B) Brightfield Analysis of RAW macrophage cells performed in control and cells chronically cultivated in carbon black (CB – 100 ug/ml, 2 weeks). (C) Cell size analyzed via brightfield microscopy with a calibrated imaging system (Nikon TE2000, Elements Software).

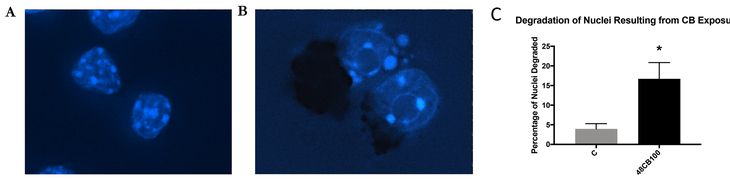


Figure 3: Proportion of degraded nuclei increases significantly after long exposure to 100 μg/mL CB in murine macrophages. Cells were seeded at density of approx. 15,000/cm² in 24-well plates. Cells were given 30 hours time for recovery and proliferation. Subsequently, media was replaced with conditional media 100 μg/mL carbon black solution (CB100) and control media was replaced with fresh DMEM. After treatment, cells were fixed in 2% PFA for 15 minutes, then gently washed 3x with ice cold DPBS. Cells were mounted to slides with DAPI Fluoromount and given 12 hours to set. Slides were sealed with clear nail polish and pictures were taken using a Nikon Eclipse Ti microscope at 460 nm. (A) Healthy control nuclei. (B) CB-stressed cells. Of note is the heterochromatin outside the nucleus with apparent nuclear blisters. (C) Degraded nuclei were counted by hand. (*=significance of $p<0.05$)

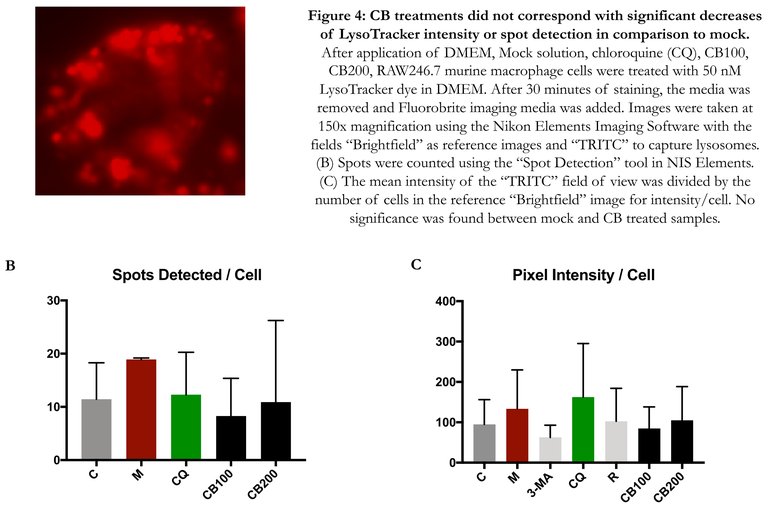


Figure 4: CB treatments did not correspond with significant decreases of LysoTracker intensity or spot detection in comparison to mock. After application of DMEM, Mock solution, chloroquine (CQ), CB100, CB200, RAW246.7 murine macrophage cells were treated with 50 nM LysoTracker dye in DMEM. After 30 minutes of staining, the media was removed and Fluorobrite imaging media was added. Images were taken at 150x magnification using the Nikon Elements Imaging Software with the fields "Brightfield" as reference images and "TRITC" to capture lysosomes. (B) Spots were counted using the "Spot Detection" tool in NIS Elements. (C) The mean intensity of the "TRITC" field of view was divided by the number of cells in the reference "Brightfield" image for intensity/cell. No significance was found between mock and CB treated samples.

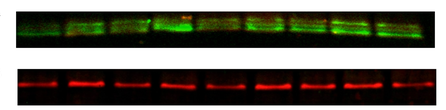


Image 2: Representative Western blot bands. 800 nm wavelength channel shows LC3-I and LC3-II at 19 and 17 kDa respectively (A) while 700 nm wavelength shows tubulin (B). Western blots were viewed with a LiCOR Imaging Scanner.

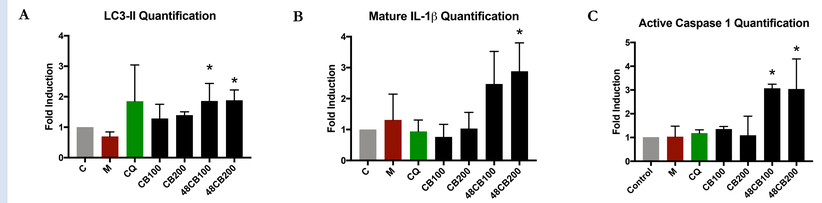


Figure 5: Western blot quantification of LC3-II, IL-1B, and Caspase 1. Raw cells were seeded onto 10 cm^2 plates at approximately 70% confluency and were treated with the following: DMEM media, Mock solution (1x BSA/PBS solution), CB100 (100 μg/ml), CB200 (200 μg/ml), 100nM rapamycin (R), and 20μM chloroquine (CQ) for 24 hours along with 3-MA (5 mM) at 12 hours and CB100 and CB200 at 48 hours after seeding (48CB100 and 48CB200 respectively). The cells were washed with ice-cold DPBS and harvested with hot SDS-Lysis buffer and triturated. Protein concentrations were determined by BCA Assay. 30 μg of lysate were loaded in a 4-20% gradient Invitrogen polyacrylamide gels and run at 110 V for 100 minutes. After transferring to a PVDF membrane with 350 mΩ for 2 hours, the membrane was blocked with a 1:3 dilution of Odyssey blocking buffer. Primary antibodies specific to the target protein of either LC3 (A), IL-1β (B), or caspase1(C) were applied at 15: 20000, then after TBST washing, fluorescent secondary antibodies were applied at 1:2000. Western blots were quantified with a LiCOR Imaging Scanner and were normalized first to tubulin and then to control. Care was taken to aliquot lysates appropriately to prevent excessive freeze-thaw cycles. Significance (* as $\alpha = 0.05$) was determined based on comparisons between mock and CB treatment groups with a one-way ANOVA with Sidak's multiple comparison post-hoc test.

Acknowledgements

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Conclusions

1. **Sonicated carbon creates a gradient of particle size.**
2. **CB activates an inflammatory response with prolonged (48 hr) exposure.**
3. **Lysosomal number and intensity decreases with an increasing concentration and exposure to CB.**
4. **Increasing CB concentration and exposure increases the number of autophagosomes.**
5. **Overall autophagic flux increases with increasing concentration and exposure to CB.**