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Original Contribution

ESR evidence for in vivo formation of free radicals in tissue of mice exposed to single-walled carbon nanotubes

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ABSTRACT

Nanomaterials are being utilized in an increasing variety of manufactured goods. Because of their unique physicochemical, electrical, mechanical, and thermal properties, single-walled carbon nanotubes (SWCNTs) have found numerous applications in the electronics, aerospace, chemical, polymer, and pharmaceutical industries. Previously, we have reported that pharyngeal exposure of C57BL/6 mice to SWCNTs caused dose-dependent formation of granulomatous bronchial interstitial pneumonia, fibrosis, oxidative stress, acute inflammatory/cytokine responses, and a decrease in pulmonary function. In the current study, we used electron spin resonance (ESR) to directly assess whether exposure to respirable SWCNTs caused formation of free radicals in the lungs and in two distant organs, the heart and liver. Here we report that exposure to partially purified SWCNTs (HiPco technique, Carbon Nanotechnologies, Inc., Houston, TX, USA) resulted in the augmentation of oxidative stress as evidenced by ESR detection of α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone spin-trapped carbon-centered lipid-derived radicals recorded shortly after the treatment. This was accompanied by a significant depletion of antioxidants and elevated biomarkers of inflammation presented by recruitment of inflammatory cells and an increase in proinflammatory cytokines in the lungs, as well as development of multifocal granulomatous pneumonia, interstitial fibrosis, and suppressed pulmonary function. Moreover, pulmonary exposure to SWCNTs also caused the formation of carbon-centered lipid-derived radicals in the heart and liver at later time points (day 7 postexposure). Additionally, SWCNTs induced a significant accumulation of oxidatively modified proteins, increase in lipid peroxidation products, depletion of antioxidants, and inflammatory response in both the heart and the liver. Furthermore, the iron chelator deferoxamine noticeably reduced lung inflammation and oxidative stress, indicating an important role for metal-catalyzed species in lung injury caused by SWCNTs. Overall, we provide direct evidence that lipid-derived free radicals are a critical contributor to tissue damage induced by SWCNTs not only in the lungs, but also in distant organs.

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Single-walled carbon nanotubes (SWCNTs) are an allotrope of carbon formed by a sheet of graphene rolled into a seamless cylinder with a diameter of 1–4 nm and a length ranging from 100 nm to 1.5 μm. SWCNTs exhibit unique electronic and mechanical properties that are used in numerous applications such as field-emission displays, nanocomposites, nanosensors, conductive plastics, paints, technical textiles and repelling features for garments, and biomedical applications. These materials are also on the leading edge of electronic fabrication and are expected to play a major role in the next generation of miniaturized electronics and in energy storage, such as hydrogen fuel cells and other efficient renewable energy sources [1–6]. However, knowledge of the potential health and environmental risks that may occur throughout the entire life cycle of products is limited [5,7].

The intrinsic toxicity of SWCNTs has been attributed to their distinctive physicochemical properties, including their small particle size and the large surface area of the carbon tube decorated with catalytic transition metals (iron, cobalt, nickel, etc.) that are known to trigger generation of free radicals and oxidative stress. Oxidative stress is one of the well-known mechanisms of SWCNT-induced toxicity [8,9]. It is believed that free radical formation by

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SWCNTs is realized via several mechanisms, frequently involving redox-cycling pathways.

There are many reports showing in vitro generation of free radicals in a number of mammalian cell types exposed to SWCNTs by employing various intracellular fluorescent dyes [10–12]. Fluorescent dye probes provide convenient, fast, and easy methods to detect oxidative stress in cells [13]. Usually these compounds are nonfluorescent (or weakly fluorescent), but yield highly fluorescent products upon reaction with free radicals. However, a major concern is the generation (or photogeneration) of free radicals by the probes themselves and/or their reaction products, which may result in artifactual results and/or higher backgrounds [14,15].

The oxidative modification of biomolecules has been systematically observed under normal and pathological conditions [16,17]. However, to understand the role of redox chemistry in the observed health outcomes and its role in the development of disease, many steps during the generation of free radicals must be linked: the identification of the biomolecule(s) that is the target of oxidative modification, the specific residue(s) at which the radicals are formed, metabolic activation targeting cellular/subcellular structures and tissue injury, and ultimately the whole-body inflammatory responses. The only tool that directly provides evidence of free radical formation is the electron spin resonance (ESR) spin trapping technique, which currently is the "gold standard" for the measurement of free radicals [16]. Previously, we and others reported employing nitrone spin trapping agents, e.g., α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN), to detect free radical species in tissues and organs of animals exposed to either chemicals [8,18–20] or airborne particulates [21,22]. The use of POBN spin trapping is a critical technical approach to identifying radical adducts (lipid and/or protein radical adducts) by extending short radical lifetimes and enabling timely recording of free radicals in many biological systems in vitro and in vivo, including tissue samples and biological fluids from living organisms.

In addition to possible direct effects of carbon nanotubes (CNTs) in the lung after inhalation/aspiration exposure, carbon nanomaterials have been shown to translocate to secondary target organs [23,24]. Pulmonary exposure to CNTs has been shown to cause dose-dependent inflammation and necrosis within the liver and kidney, reductions in the serum antioxidant capacity, indirect thrombogenic effects within the liver and heart, microvascular dysfunction, and possible effects on cardiac autonomic regulation [24–30]. Mercer and colleagues [31] demonstrated that multi-walled carbon nanotubes (MWCNTs) deposited in the lungs were transported to the pleura, respiratory musculature, liver, kidney, heart, and brain. Matthews et al. [32] examined and predicted cumulative pulmonary translocation of SWCNTs from the lung airways of rats using an ex vivo isolated perfused-lung model.

Here for the first time we provide evidence for in vivo generation of carbon-centered free radicals in mouse lungs, heart, and liver after exposure to respirable SWCNTs. We demonstrate that the carbon-centered lipid-derived radicals detected are an intermediate of enhanced lipid peroxidation in the lung, then later in the heart and liver, in response to SWCNTs. Pulmonary exposure to SWCNTs induced oxidative stress and significantly reduced the level of antioxidants in mouse lungs, facilitating recruitment of inflammatory cells and increasing the profile of proinflammatory cytokines in the bronchoalveolar lavage (BAL) fluids. However, at a later time point (7 days postexposure), SWCNTs triggered a significant accumulation of oxidatively modified protein carbonyls, an increase in lipid peroxidation products, a decrease in protein thiols, the depletion of glutathione (GSH), and signs of inflammation only in the heart and liver. Additionally, the metal chelator deferoxamine (DFO) significantly inhibited biomarkers of inflammation and oxidative stress in the lung, demonstrating an important role for metal-catalyzed species in lung injury caused by SWCNTs.

Methods

Animals and treatments

Specific-pathogen-free adult female C57BL/6 mice (7–8 weeks of age) were supplied by The Jackson Laboratory (Bar Harbor, ME. USA) and weighed 20.3 ± 0.21 g at the time of use. Animals were individually housed in AAALAC-approved NIOSH animal facilities in microisolator cages for 1 week before use. Autoclaved Beta Chip bedding (Northeastern Products Corp., Warrensburg, NY, USA) was changed weekly. Animals were supplied with water and Harlan Teklad, 7913, NIH-31 Modified Mouse/Rat Diet, Irradiated (Harlan Teklad, Madison, WI, USA) and housed under controlled light, temperature, and humidity conditions. Experiments were conducted under a protocol approved by the Animal Care and Use Committee of the National Institute for Occupational Safety and Health.

A suspension of SWCNTs (40–80 μg/mouse) was used for a single pharyngeal aspiration of C57BL/6 mice, whereas the corresponding control mice were administered a sterile $Ca^{2+}+Mg^{2+}$-free phosphate-buffered saline (PBS) vehicle. Mice were sacrificed on days 1 and 7 after exposure. For each group, six animals were used to evaluate BAL (cell differential and cytokine accumulation), fibrogenic responses, tissue damage, inflammation, and oxidative stress markers and to conduct histopathology. To study free radical generation, POBN (6 mmol/kg) was injected intraperitoneally (ip) 30 min before tissue collection. To investigate whether hydroxyl radicals were produced in the lungs of SWCNT-treated mice, the ^{13}C-labeled dimethyl sulfoxide ([^{13}C]DMSO, 1 ml/kg) was administered together with POBN to another group of mice. Lipid extracts of the lungs, liver, and heart were used to measure radical adduct content. An additional group of mice was pretreated with DFO (100 mg/kg, ip, 2 and 24 h before pharyngeal aspiration of SWCNTs). Inflammation and oxidative stress markers were measured in the lung homogenates of mice sacrificed 24 h after exposure to SWCNTs.

Particles

The SWCNTs (Carbon Nanotechnologies, Inc., Houston, TX, USA) used in this study were produced by the high-pressure CO disproportionation process (HiPco) technique [33], employing CO in a continuous-flow gas phase as the carbon feedstock and Fe $(CO)_5$ as the iron-containing catalyst precursor, and purified by acid treatment to remove metal contaminants [34]. The particles were characterized by chemical analysis using the NIOSH *Manual of Analytical Methods* No. 5040 and inductively coupled plasma atomic emission spectroscopy. SWCNTs were 99.7% wt elemental carbon with 0.23% wt iron. Individual SWCNTs had diameters ranging from 1 to 4 nm and were 1–3 μm in length. SWCNTs were found to have a specific surface area of 1040 m^2/g. Detailed particle characterization was published in [44]. Before animal exposure, particles were ultrasonicated (30 s × 3 cycles) on ice for improved dispersion of nanoparticles using a Vibra Cell (Sonics and Materials, Newtown, CT, USA) probe sonicator operating at 20 kHz (6.5% power).

Particulate instillation

Mouse pharyngeal aspiration was used for particulate administration. Briefly, after anesthetization with a mixture of ketamine (Phoenix, St. Joseph, MO, USA) and xylazine (Phoenix) (62.5 and 2.5 mg/kg subcutaneously in the abdominal area), the mouse was placed on a board in a near-vertical position and the animal's tongue extended with lined forceps. A suspension (approximately 50 μl) of particulates prepared in PBS with SWCNTs at a dose of 0,