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Lysosomal membrane permeabilization: Carbon nanohorn-induced reactive oxygen species generation and toxicity by this neglected mechanism

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ABSTRACT

Understanding the molecular mechanisms responsible for the cytotoxic effects of carbon nanomaterials is important for their future biomedical applications. Carbon nanotubular materials induce the generation of reactive oxygen species (ROS), which causes cell death; however, the exact details of this process are still unclear. Here, we identify a mechanism of ROS generation that is involved in the apoptosis of RAW264.7 macrophages caused by excess uptake of carbon nanohorns (CNHs), a typical type of carbon nanotubule. CNH accumulated in the lysosomes, where they induced lysosomal membrane permeabilization (LMP) and the subsequent release of lysosomal proteases, such as cathepsins, which in turn caused mitochondrial dysfunction and triggered the generation of ROS in the mitochondria. The nicotinamide adenine dinucleotide phosphate oxidase was not directly involved in CNH-related ROS production, and the ROS generation cannot be regulated by mitochondrial electron transport chain. ROS fed back to amplify the mitochondrial dysfunction, leading to the subsequent activation of caspases and cell apoptosis. Carbon nanotubules commonly accumulate in the lysosomes after internalization in cells; however, lysosomal dysfunction has not attracted much attention in toxicity studies of these materials. These results suggest that LMP, a neglected mechanism, may be the primary reason for carbon nanotubule toxicity.

Introduction

Carbon nanotubular materials, including carbon nanotubes and carbon nanohorns (CNHs), have attracted considerable interest and are promising materials for drug delivery (Liu et al., 2011; Ajima et al., 2005; Murakami et al., 2008), imaging (Miyawaki et al., 2006; Liu et al., 2009) and photodynamic therapy (Zhang et al., 2008; Moon et al., 2009). However, once they are engulfed by cells, the effects of carbon nanotubules on the function of sub-cellular organelles and biomolecules are not fully understood. To permit practical applications of carbon nanotubular materials in life sciences, extensive toxicological studies are necessary.

Recently, more and more studies show that carbon nanotubular materials induce cell apoptosis as indicated by peroxidative product generation, the expression of signals such as p53 (Ravichandran et al., 2010), bax (Cui et al., 2005; Ravichandran et al., 2010), NF-κB, and AP-1 (Ravichandran et al., 2010; Pacurari et al., 2008), caspase activation (Ravichandran et al., 2009), and antioxidant depletion (Ravichandran et al., 2009; Srivastava et al., 2011; Wang et al., 2011). Among these studies, most report that oxidative stress and the production of reactive oxygen species (ROS), which are involved in the apoptosis pathway (Cheng et al., 2011), are the major adverse effects mediated by carbon nanotubules (Ravichandran et al., 2009; Srivastava et al., 2011; Pacurari et al., 2008; Lacotte et al., 2008; Liu et al., 2013; Yang et al., 2009). Although some previous reports discussed ROS generation by carbon nanotubules (Kagan et al., 2006; Lacotte et al., 2008; Sharma et al., 2007; Pulskamp et al., 2007; Ye et al., 2011), a detailed understanding of the mechanisms and pathways involved is currently lacking.

Abbreviations: CNHs, carbon nanohorns; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; LMP, lysosomal membrane permeabilization; AO, acridine orange; BA_1, bafilomycin A_1; TEM, transmission electron microscopy; NAC, N-acetyl-L-cysteine; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; H_2DCF, 2′,7′-dichlorodihydrofluorescein; DCF, 2′,7′-dichlorofluorescein; $\Delta\psi_m$, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolyl carbocyanine iodide; PCN, pyocyanin; Rot, rotenone; Ant A, antimycin A; Apo, apocynin; PA, pepstatin A; AP, antipain dihydrochloride; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; AD, actinomycin D; ETC, electron transport chain; MWNT, multi-walled carbon nanotube; SWNT, single-walled carbon nanotube; Cyt C, cytochrome C.

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In this study, we found that the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was not directly involved in CNH-related ROS production, and the mitochondrial electron transport chain (ETC) cannot regulate the ROS generation induced by CNHs. CNHs caused lysosomal membrane permeabilization (LMP), and the subsequent release of cathepsins from the lysosomes triggered the generation of ROS. These results indicated that lysosomal dysfunction may be the primary reason for ROS generation and cell death. Although carbon nanotubular materials are generally localized in the lysosomes (Porter et al., 2007; Yang et al., 2010), lysosomal dysfunction has not attracted much attention in toxicity studies of these materials. Our results suggest that CNHs and, may be also other carbon nanotubular materials, induce LMP after entering the cells and LMP is a neglected mechanism of nanotubule-induced cell death and may be one of the main reasons for carbon nanotubule-induced toxicity.

In this study, we used CNHs, because they have a uniform structure, with a diameter of 2–5 nm and a length of 40–50 nm, and they assemble together to form a spherical aggregate with a diameter of approximately 100 nm. Additionally, they do not contain metal impurities because the production of CNHs does not require metal catalysts (Iijima et al., 1999). These properties reduce considerably the possibility of conflicting results caused by variations in particle size, shape, and metal impurity (Liu et al., 2013; Sohaebuddin et al., 2010). Similar to other carbon nanotubules, CNHs often accumulate in macrophages (Tahara et al., 2011; Miyawaki et al., 2009); therefore, we examined the molecular mechanisms underlying CNH-induced apoptosis in RAW264.7 macrophages. The effects of the CNH dose and exposure time were investigated, and the link between LMP and ROS generation, especially which is the initial stage, and cell apoptosis is discussed. Recently, lysosomal dysfunction was proposed as a mechanism of nanomaterial toxicity (Stern et al., 2012). The results of this study provide clues to understand how carbon nanotubules induce the production of ROS and subsequent cell death.

Materials and methods

General

Murine RAW264.7 macrophages from the European Collection of Cell Cultures were cultured as a monolayer at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were maintained in RPMI medium 1640 (Life Technologies, USA) containing 10% fetal bovine serum (Life Technologies, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, USA). 0.25% Trypsin–EDTA, Triton X-100, CelLytic-M reagent, Bradford reagent, acridine orange, rotenone, antimycin A, apocynin, NAC, pepstatin A, antipain dihydrochloride, JC-1, and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

The as-grown CNHs were produced by CO_2 laser ablation of pure graphite in an argon atmosphere (1 atm) at room temperature without metal catalysts (Iijima et al., 1999). The purity was estimated to be 95%, with 5% giant graphite ball particles (Azami et al., 2008). The as-grown CNHs were treated with light-assisted oxidation, using hydrogen peroxide to introduce abundant carboxyl groups, as described previously (Zhang et al., 2007; for details see Section 1 in Supplementary Information). The resulting products (referred to as CNH_{Laox}) were dispersed in deionized water by sonication for 10 min in a bath-type sonicator. The CNH_{Laox} water solution was then centrifuged at 2580 *g* for 10 min to remove large aggregates. The concentration of CNH_{Laox} in the supernatant was determined using a Lambda 1050 UV–vis-NIR spectrometer (PerkinElmer, USA) based on the visible-light absorption of CNH_{Laox} at 700 nm. CNH_{Laox} was reconstituted in cell culture medium to concentrations of 1, 10, 50, and 100 μg/ml, and these CNH_{Laox} dispersions were used in the subsequent cell experiments.

Cellular uptake of CNH_{Laox}

After treatment with 1–100 μg/ml CNH_{Laox} for 24 or 48 h, the cellular uptake amount of CNH_{Laox} was estimated from the light absorption intensity (700 nm) of cell lysates, as reported previously (Zhang et al., 2012; for details, see Section 2 in Supplementary Information).

To determine the intracellular localization of CNH_{Laox}, cells (2×10^5 cells/ml) incubated with CNH_{Laox} (10 μg/ml) on glass-bottomed dishes were washed twice with PBS, and incubated in a fresh medium containing 50 nM LysoTracker red DND-99 (Life Technologies, USA) for further 20 min before being observed under a LSM 5 PASCAL confocal microscope (Carl Zeiss Inc., Germany).

Cytotoxicity assay

The cytotoxicity of CNH_{Laox} to RAW264.7 cells was determined using the cell proliferation reagent WST-1 (Roche Diagnostics, Switzerland) and a Bradford protein assay, as described previously (Yang et al., 2013; for details, see Section 3 in Supplementary Information).

Acridine orange (AO) release

After incubation with CNH_{Laox} or N-acetyl-L-cysteine (NAC) for different time periods at 37 °C, the cells were rinsed twice with PBS and stained with 3 ml of 5 μg/ml AO for 15 min at 37 °C. Then the detached cells were suspended in 3 ml of the cell medium and analyzed using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Inc., USA) at an excitation wavelength of 488 nm. Emission was recorded at 525 nm for green AO fluorescence and at 620 nm for red AO fluorescence.

For the confocal microscopy observations, the cells were incubated with CNH_{Laox} on glass-bottomed dishes, washed twice with PBS, stained with 5 μg/ml AO for 15 min at 37 °C, and washed three times with PBS. The stained cells were observed under a LSM 5 PASCAL confocal microscope (Carl Zeiss Inc., Germany).

Electron microscopy

To observe the morphological changes of lysosomal membrane, CNH_{Laox} treated or untreated RAW264.7 cells were prefixed for 1 h at room temperature in 1.2% glutaraldehyde in 0.1 M pH 7.2 phosphate buffer, postfixed in 1% osmium tetroxide for 1 h at room temperature, dehydrated in ethanol, substituted with n-butyl glycidyl ether and Epon 812, and embedded in Epon 812. Thin sections were counterstained with 2% uranyl acetate and 0.5% lead citrate, and they were then observed by a H-7600 transmission electron microscopy (TEM, 80 kV, Hitachi, Ltd., Japan).

Detection of cathepsin D by immunofluorescence studies

The cells were incubated with CNH_{Laox} on glass-bottomed dishes, washed twice with PBS, fixed with 3% paraformaldehyde in PBS for 30 min at 4 °C, and blocked in 10% goat serum in PBS that contained 0.3 M glycine. The cells were incubated with a monoclonal rabbit anti-cathepsin D antibody (dilution 1:50, GeneTex Inc., USA) at 4 °C overnight, followed by a goat anti-rabbit IgG-Alexa fluor 488 antibody (dilution 1:500, Life Technologies, USA) after washing three times with 1% goat serum in PBS. Thereafter the cells were rinsed in 1% goat serum PBS three times again and examined under a LSM 5 PASCAL confocal microscope (Carl Zeiss Inc., Germany).