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Inhibition of catecholamine secretion by iron-rich and iron-deprived multiwalled carbon nanotubes in chromaffin cells

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ABSTRACT

The assay of the toxic effects of carbon nanotubes (CNTs) on human health is a stringent need in view of their expected increasing exploitation in industrial and biomedical applications. Most studies so far have been focused on lung toxicity, as the respiratory tract is the main entry of airborne particulate, but there is also recent evidence on the existence of toxic effects of multiwalled carbon nanotubes (MWCNTs) on neuronal and neuroendocrine cells (Belyanskaya et al., 2009; Xu et al., 2009; Gavello et al., 2012). Commercial MWCNTs often contain large amounts of metals deriving from the catalyst used during their synthesis. Since metals, particularly iron, may contribute to the toxicity of MWCNTs, we compared here the effects of two short MWCNTs samples (<5 μm length), differing only in their iron content (0.5 versus 0.05% w/w) on the secretory responses of neurotransmitters in mouse chromaffin cells.

We found that both iron-rich ($MWCNT_{+Fe}$) and iron-deprived ($MWCNT_{-Fe}$) samples enter chromaffin cells after 24 h exposure, even though incorporation was attenuated in the latter case (40% versus 78% of cells). As a consequence of $MWCNT_{+Fe}$ or $MWCNT_{-Fe}$ exposure (50–263 μg/ml, 24 h), catecholamine secretion of chromaffin cells is drastically impaired because of the decreased Ca^{2+}-dependence of exocytosis, reduced size of ready-releasable pool and lowered rate of vesicle release. On the contrary, both MWCNTs were ineffective in changing the kinetics of neurotransmitter release of single chromaffin granules and their quantal content. Overall, our data indicate that both MWCNT samples dramatically impair secretion in chromaffin cells, thus uncovering a true depressive action of CNTs mainly associated to their structure and degree of aggregation. This cellular "loss-of-function" is only partially attenuated in iron-deprived samples, suggesting a minor role of iron impurities on MWCNTs toxicity in chromaffin cells exocytosis.

1. Introduction

Carbon nanotubes (CNTs) are cylindrical structures composed of carbon atoms, driving the attention of the scientific community because of their unique physico-chemical properties. Given their potential wide use in many fields, from industry to biomedicine, it is fundamental to reveal their potential hazard. Most of the commercial CNTs contain ultrafine metal particles derived from the original growth catalyst such as Fe, Ni, Y, Co (Liu et al., 2008). Metal induced adverse effects are a real concern regarding most CNTs presently available on the market, since many studies reported that bioavailable metal impurities play a major role in CNTs-induced cytotoxicity. New purification methods could be thus a promising approach to prevent their toxicity (Liu et al., 2008). One of the main techniques used to purify CNTs surface from metal impurities is the treatment using different inorganic acids. As proposed by Nimmagadda et al. (2006), the treatment of SWCNTs with 2 M nitric acid for 10 h significantly improves CNTs biocompatibility, reducing the amount of the catalyst residues. The main aspect to consider is that purification technologies may alter the nanotube structure (Martinez et al., 2003). Thus, the chemical composition of each nanotube preparation is of crucial importance for biological applications. Here we took advantage of a treatment

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with the non-oxidizing acid HCl (Liu et al., 2008), which reduces the quantity of iron impurity preserving the size and degree of nanotubes crystallinity, to assay the effects of MWCNTs on chromaffin cell secretion.

Cellular uptake and cytotoxic impact of CNTs are the main issues explored in the literature. In particular, it would be useful to develop new specific cytotoxicity assays for the rapid and reliable screening of the multitude of CNT types fabricated today (Ali-Boucetta et al., 2011). However, little is known on the effects of SWCNTs and MWCNTs on cell functions, especially on neuronal and neuronal-like preparations. In previous findings we reported that exposure of mouse chromaffin cells to MWCNTs impairs the spontaneous activity without affecting Na^+, Ca^{2+} and K^+ conductances (Gavello et al., 2012), however reports on the effects of CNTs on the functionality of the secretory apparatus are almost absent in the literature. It is proved that both single and multiwalled CNTs induce actin filaments and VE-cadherin disruption, cytotoxicity and reduced tubule formation after 24 h exposure in human aortic endothelial cells (Walker et al., 2009), but it is not known if this happens also in other cell types such as neurons, and how this could affect secretory vesicles trafficking. Malarkey et al. (2008) reported that water soluble SWCNTs inhibit stimulated endocytosis in rat hippocampal cultures: the long length of the nanotube seems to prevent the vesicle from closing and pinching off from the membrane, inhibiting the endocytosis.

Here we investigated the effects of well characterized MWCNTs on neuroendocrine chromaffin cells. In particular, we compared two different samples of short MWCNTs (<5 μm length): the iron-rich ($MWCNT_{+Fe}$) and the iron-deprived sample ($MWCNT_{-Fe}$). In the latter sample the amount of iron impurities was dramatically reduced by treatment with HCl, leaving unaltered the size and crystallinity of the nanotubes. Our study is focused on the inhibitory action of MWCNTs (50–263 μg/ml) on the exocytotic properties of adrenal chromaffin cells, representing a suitable model of neuronal-like excitable cells which can massively secrete adrenaline and noradrenaline during "fight-or-flight" responses (Carabelli et al., 2003, 2007; Giancippoli et al., 2006). Both MWCNT samples can translocate into the cells, as revealed by the presence of the MWCNTs into the cytoplasm and the nucleus, thus causing a marked reduction of exocytosis without affecting Ca^{2+} channels activity, as previously reported (Gavello et al., 2012). Interestingly, the toxic effect on secretion is not abolished but only partially attenuated by iron-deprived samples ($MWCNT_{-Fe}$). This suggests that the main depressive effect of MWCNTs is likely attributed to either their intrinsic toxicity or their degree of aggregation and not to iron impurities released from $MWCNT_{+Fe}$. Furthermore, the amount of iron released after cell incubation with $MWCNT_{+Fe}$ raises by only 4% of the total iron normally present in the control culture media (from 9.4 μM to 9.8 μM), which is far below the concentration expected to produce significant effects on cell excitability (1–2 mM, Lopin et al., 2012).

2. Materials and methods

2.1. Isolation and culture of adrenal medulla chromaffin cells

Mouse chromaffin cells (MCCs) were obtained from young C57BL/6J male mice (Harlan, Milano, Italy). All experiments were conducted in accordance with the guidelines on Animal Care established by the Italian Minister of Health and approved by the local Animal Care Committee of Turin University.

After removal, the adrenal glands were placed in Ca^{2+} and Mg^{2+} free Locke's buffer, containing (mM): 154 NaCl, 3.6 KCl, 5.6 $NaHCO_3$, 5.6 glucose, and 10 HEPES (pH 7.3, at room temperature). Glands were then decapsulated to separate the medullas from the cortex. Enzymatic digestion was achieved by keeping the medulla for 20 min at 37 °C into a DMEM solution enriched with 0.16 mM L-cysteine, 1 mM $CaCl_2$, 0.5 mM EDTA, 20 U/ml of papain (Worthington Biochemical, Lakewood, NJ, USA), 0.1 mg/ml of DNAse (Sigma, Milan, Italy). The digested glands were then washed with a solution containing DMEM, 1 mM $CaCl_2$, 10 mg/ml BSA and resuspended in 2 ml DMEM supplemented with 15% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA). Isolated chromaffin cells were obtained after mechanical disaggregation of the glands. Cells were then incubated at 37 °C in a water-saturated atmosphere with 5% CO_2 and used within 2–4 days after plating.

Rat chromaffin cells were prepared for electron microscopy trials. The culture was prepared as explained before for mouse chromaffin cells, with the exception of the enzyme papain, which was replaced with a solution of liberase-blendizymes-3 at the concentration of 0.35 mg/ml (Roche, Switzerland). Thus, 100 μl of liberase solution were added for each ml of DMEM (Carabelli et al., 2007).

2.2. Preparation of MWCNTs

Pristine multi walled carbon nanotubes ($MWCNT_{+Fe}$) were purchased by Mitsui Chemicals (Kawasaki-Shi, Japan); this sample was then shortened by grinding in an oscillatory agate ball mill for 6 h. One aliquot of was suspended in 1 M HCl to dissolve iron impurities and the suspension stirred at room temperature for 10 days. These obtained purified MWCNTs ($MWCNT_{-Fe}$) were then recovered by centrifugation, washed with distilled water and dried (Liu et al., 2008).

2.3. Physico-chemical characterization

2.3.1. Surface area

The surface area of MWCNTs was measured by means of the BET method based on N_2 adsorption at −196 °C (Micrometrics ASAP 2010).

2.3.2. Morphology

The dimension of MWCNTs has been evaluated by means of atomic force microscopy (AFM) and transmission electron microscopy (TEM). The morphology and the statistical evaluation of diameters and lengths of the MWCNT fragments were obtained by considering a population of 300 data each, coming from AFM scans (10×10, 5×5 and/or 2×2 μm in size) and from low resolution TEM (4000× and/or 25,000×) of the ground sample before and after the removal of iron. AFM images of nanotubes were collected in non-contact mode by a Park Systems XE-100, while TEM images were collected by a JEOL 3010-UHR TEM instrument operating at 300 kV. As for the sample preparation, MWCNTs were dispersed in isopropyl alcohol and a droplet of the diluted solution was placed onto a freshly cleaved mica substrate or a TEM grid.

2.3.3. Elemental analysis

Metallic contamination was quantified after calcination of the different MWCNT preparations and re-suspension of the residue in concentrated HCl. The concentration of iron in the solution was quantified. The analysis of the obtained solution was performed by atomic emission-inductively coupled plasma (AE-ICP) spectrometry (IRIS II Advantage/1000, Thermo Jarrel Ash, Franklin, MA).

2.3.4. Structural analysis

Micro-Raman spectra were acquired using an integrated micro/macro Raman system which includes a Horiba Jobin Yvon HR800 microspectrometer, an Olympus BX41 microscope and a CCD