Communication **Open Access**

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Role of single walled carbon nanotubes (SWCNTs) on the airway smooth muscle

DOI 10.1515/mesbi-2016-0013
Received September 7, 2016; revised October 17, 2016; accepted November 17, 2016

Abstract: The aim of this work was to evaluate the effects induced by single walled carbon nanotubes (SWCNTs) on the airway smooth muscle tone, using an isolated rat tracheal rings model and monitoring nitric oxide (NO) as a possible mediator involved in the effects. Exposure of tracheal rings to SWCNTs did not modify the smooth muscle tone *per se*; however, when the rings were pre-treated with the contractile agent acetylcholine (ACh), all SWCNTs concentrations (0.1-10 μg/mL) induced a transient contractile effect similar to that induced by ACh alone. Interestingly, the NO production was not modified by SWCNTs regardless of the presence of ACh 10 μM. Thus, the data suggest that NO is not involved in the airway smooth muscle contraction induced by SWCNTs. Further investigations are required to understand the effects, mediator(s) and mechanisms of action induced by this type of porous nanomaterial, as well as their fine interactions with the respiratory system structures.

Keywords: Nanomaterials, SWCNTs, trachea, hyperreactivity

1 Introduction

Nanotechnology has become a promising field in the generation of new medical treatments using nanomaterials (NMs). These structures are reported in the literature with at least one dimension in the range of 1-100 nm and both the size and material nature define their physical and chemical properties (Chen & Schluesener, 2008). Among all NMs with potential biological applications, carbon nanotubes (CNTs) are one of the most studied (Wu et al., 2008). CNTs are porous material composed of carbon sp^2 hybridized arranged in a three dimensional hexagonal molecule conforming rolled-up sheets of graphene. CNTs composed of a single graphene sheet are called single walled carbon nanotubes (SWCNTs) (Sharma et al., 2016), which have shown potential biomedical applications (Liu et al., 2009; Yang et al., 2010; Sridharan et al., 2009). SWCNTs involve an important risk of interaction with the human body through various routes of exposure, such as the respiratory system. It has been demonstrated that SWCNTs can deposit in the lungs and induce the release of inflammatory mediators (Erdely et al., 2009). In this concern, the nitric oxide (NO) is a versatile molecule related with nanomaterials-induced inflammation (Nurkiewicz and Porter, 2006; Courtois et al., 2008), promoting airway inflammatory diseases associated with hyperreactivity processes. These processes are defined as an excessive bronchial narrowing and manifest as an exaggerated bronchoconstriction in response to different inhaled stimuli (Grootendorst and Rabe, 2004). In this sense, adverse effects of CNTs on the respiratory system were observed in various experimental models (Pacurari et al., 2016; Erdely et al., 2009). Nevertheless, these models do not provide relevant information regarding the mechanisms of action, particularly those related to the airway muscle tone (contraction and relaxation) and responsible for regulating the airflow in the respiratory system (James and Carroll, 2000). Our research group found that SWCNTs induced a NO-independent relaxation in the aortic vascular smooth muscle, in an inverse dose-dependent relationship. The lowest SWCNTs concentration induced a marked vasodilation; meanwhile the highest dose elicited a slight dilator effect, suggesting that a contractile effect could be exerted at high SWCNTs concentrations (Gutierrez-Hernandez et al., 2015). Considering these findings, SWCNTs could affect the tone of smooth muscle, such as airway

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smooth muscle. The aim of this study was to evaluate in an isolated rat tracheal rings model the physiological effects induced by SWCNTs in the airway smooth muscle tone as well as the role of NO as a possible mediator.

2 Experimental Procedures

2.1 Chemicals

SWCNTs were purchased from Thomas Swan, Advanced Materials (United Kingdom). Other compounds, such ACh (>99%) and various salts were purchased from Sigma Chemical Company (St. Louis, MO.USA).

2.2 SWCNTs purification process

A batch of SWCNTs raw material was heated at 400 °C in air for 30 min. Loss of mass was insignificant. Then, the material was treated with a 12 M HCl solution for 12 h. Later, the material was centrifuged and rinsed with deionized water several times until mild acid reaction and finally the solution was filtered through a 5 μm polytetrafluoroethylene membrane filter (Zhao et al., 2003). This step was carried out before the evaluation of the physiological effects of the SWCNTs.

2.3 Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) analysis

Characterization of both crude and purified SWCNTs was conducted in FE-SEM system (FEI Inspect F50) at 25 kV of voltage and magnification of 100,000x. Samples were directly analyzed from the powder placed on the carbon tape of the microscope. With the same system, an EDS analysis of the SWCNTs elemental composition was performed before and after purification processes.

2.4 SWCNTs dispersion

Stock suspension of SWCNTs was prepared in sterile deionized water at 1.0 mg/mL and sonicated for 1 h at 60 °C and 45 kHz using a Cole-Parmer 470 50 W sonicator. Different suspensions of various SWCNTs concentrations (0.1 μg/mL, 1.0 μg/mL and 10.0 μg/mL) were prepared using sterile deionized water (Frame et al. 2014).

2.5 Turbidimetry measurements

Turbidimetry measurements were performed in an UV-vis spectrometer (Ocean Optics Model RedTide) at 633 nm in all SWCNTs concentrations used (0.1, 1.0 and 10.0 μg/mL) (Kojima, et al. 2011) to estimate the SWCNTs stability in physiological solution.

2.6 Smooth muscle tone of rat tracheal rings

Adult male Wistar rats (300–350 g weight) were sacrificed by an overdose injection of sodium pentobarbital (70 mg/kg) in accordance with animal protocols approved by the Animal Care and Use Committee of the Universidad Autonoma de San Luis Potosi. Experiments were performed as previously described (Gonzalez et al., 2011). Briefly, upon sacrifice, the trachea was excised, the epithelium was removed by gently rubbing with a cotton swab, cleansed of adhering tissue, and cut in 3–4 mm wide segments. Individual tracheal rings were suspended from a Radnoti isometric transducer in oxygenated tissue baths containing HEPES-buffered Krebs-Henseleit (KH) solution at 37 °C [118 mM NaCl, 4.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.75 mM $CaCl_2$, 20 mM $C_8H_{18}N_2O_4S$ (4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid, HEPES) and 11.1 mM glucose, 3 μM indomethacin, pH 7.4]. A passive load of 2 g was applied, and the trachea segments were allowed to equilibrate for 1 h. Rat tracheal rings were precontracted with 10 μM ACh, and then exposed to different concentrations of SWCNTs (0.1–10 μg/mL). ACh treatment was used as a positive control of smooth muscle contraction. Real-time data were collected and analyzed using Poliview software (ADI Instruments, Colorado Springs, CO).

2.7 Nitric oxide production

The oxidation products of NO, nitrite (NO_2), and nitrate (NO_3) were quantified by the Griess reaction (Kelm and Schrader, 1988; Gonzalez et al., 2011). NO products were determined in tracheal ring samples collected in KH solution after completing treatments. Absorbance was measured in a microplate reader using a 540 nm filter (Bio-Rad, Hercules, CA, USA). Assay sensitivity was 1 μM, and the standard NO curve was obtained with NO standards with concentrations ranging from 1 to 200 μM.

2.8 Statistical analysis

Data were expressed as a mean±standard error measurement of three independent experiments; they were subjected to a statistical analysis by one-way ANOVA followed by Dunnett test for multiple comparisons. Values of $p < 0.05$ were considered significant.

3 Results

3.1 SWCNTs characterization

Morphology of SWCNTs was examined using FE-SEM before and after the purification process (Fig. 1A and 1B, respectively). Purified SWCNTs possessed fewer bundles and carbonaceous impurities as well as clean surfaces in comparison to the non-purified SWCNTs, confirming an efficient purification process. In addition, elemental analysis revealed quantification of impurities (Fig. 1C). Initial EDS analysis results indicated that the main elements conforming SWCNTs were carbon and iron (93% and 2%, respectively). The amount of other carbon forms, such as carbon nanoparticles and pieces of graphitic carbon were about 4%. After the purification process, a new characterization showed that the amount of carbon increased to about 98% in the samples, indicating a higher SWCNTs purity; meanwhile carbon impurities were not detected. Furthermore, 1% and 0.50% of oxygen and chlorine, respectively, were found in the samples. These elements could be detected as a result of the purification treatment with hydrochloric acid. Iron in the SWCNTs samples was almost undetectable (Fig. 1C.I and 1C.II).

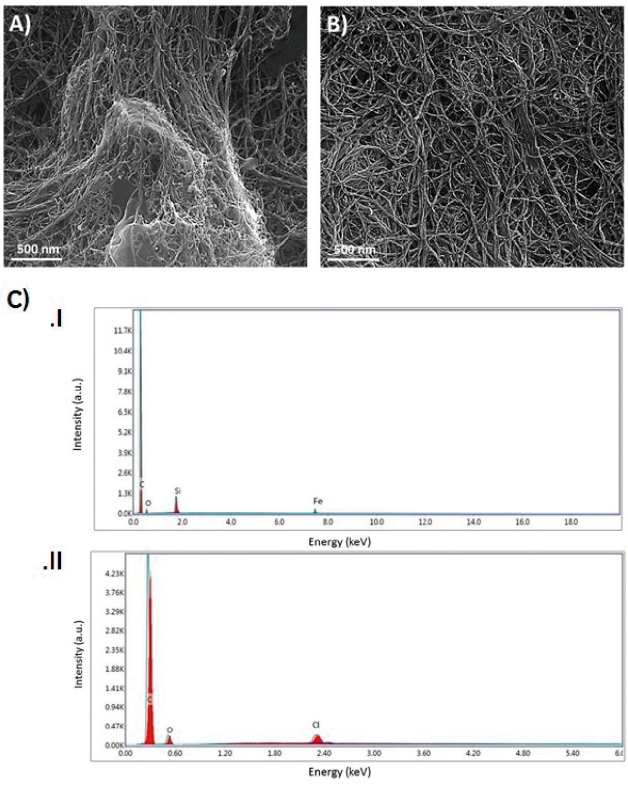

Figure 1: SEM images of SWCNTs before and after acid treatment. Large bundles of crude SWCNTs and carbonaceous impurities are noticeable (A). After purification process SWCNTs show increased dispersion and the diameter appears to be uniform without carbonaceous remnants (B). EDS spectra before (I) and after (II) purification process (C).

3.2 Stability of SWCNTs

Turbidity analysis was performed to evaluate the stability of the SWCNTs in KH solution. Stability of SWCNTs suspensions at 0.1 and 1.0 µg/mL remained for 30 min, whereas the highest concentration (10.0 µg/mL) of SWCNTs affected their stability, probably due to quick coagulations and sedimentations of SWCNTs (Fig. 2). These results suggest that SWCNTs suspensions were stable at concentrations below 10.0 µg/mL. Therefore, the physiological experiments were conducted in a range of concentrations from 0.1 to 10 µg/mL SWCNTs.

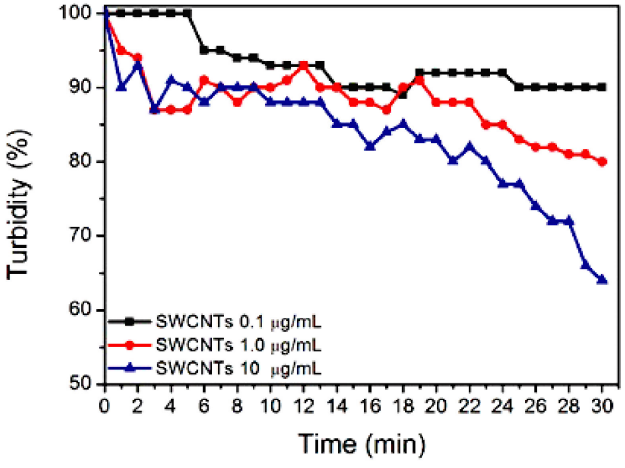

Figure 2: Stability test performed by transmission measurements using a 633 nm wavelength.In the KH solution, the turbidity of the lowest concentration SWCNTs suspension (0.1 µg/mL) was almost constant for 30 min, whereas the turbidity of the SWCNTs at the highest concentration (10.0 µg/mL), immediately decreased.

3.3 SWCNTs induced effects on trachea rings

To evaluate the direct effects of SWCNTs on the airway smooth muscle tone, tracheal rings were treated with increasing concentrations of SWCNTs ranging from 0.1 to 10 µg/mL. Solutions were administered directly into the organ baths containing the rings. The results revealed that none of the cumulative concentrations of SWCNTs modified the smooth muscle tone by themselves (Fig. 3). On the other hand, administration of single doses of SWCNTs in pre-contracted trachea rings with ACh 10 µM, induced a transient contraction evidenced as an increase in the basal tension. This effect was similar to the ACh-induced effect, but in a minor magnitude (Fig. 4A-C). The contraction magnitude was normalized using the ACh treatment as 100% of contraction. Analyzed data showed that there was no significant difference between the percentages of contraction induced by all SWCNTs concentrations. Therefore, the SWCNTs-induced effect did not depend on the concentration (Fig. 4D).

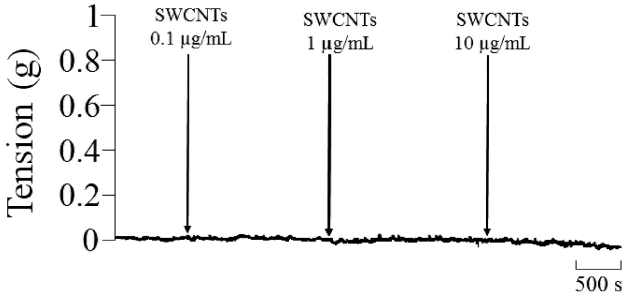

Figure 3: Cumulative concentrations of SWCNTs did not modify the trachea smooth muscle tone. Cumulative concentrations of SWCNTs (0.1, 1, 10 and 100 µg/mL) did not alter the tracheal smooth muscle contraction. The presented recording is representative of three independent experiments.

3.4 The SWCNTs-induced contraction effect is independent of NO production

NO is a versatile agent that exerts a dual role (dilator/contractile) in the smooth muscle. In the respiratory system, NO is synthesized by the constitutively expressed enzyme endothelial NO-synthase (eNOS) and neuronal synthase (nNOS), or by the inducible isoform (iNOS), under inflammation or cellular damage events (Capettini et al., 2010; Conti et al., 2013). In this study, the production of NO was determined in the KH solution that bathed tracheal rings before and after the SWCNTs exposure. In this

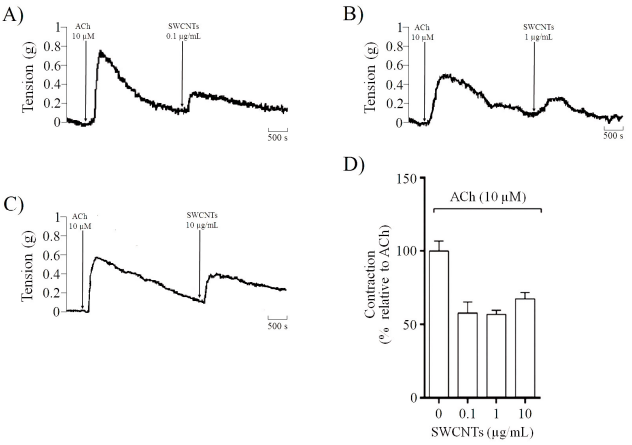

Figure 4: Effects induced by single doses of SWCNTs in the trachea rings. The rat traches rings were pre-contracted with ACh 10 µM and then, rings were treated in presence of a single concentration of SWCNTs: 0.1 (A), 1 (B) and 10 µg/mL (C) . These recordings are representative of three independent experiments. Percentage of contraction induced by SWCNTs (0.1, 1, 10 µg/mL) on pre-contracted trachea rings with ACh 10 µM was calculated as the percentage of tension based on 100% contraction induced by ACh 10 µM. Values are represent as mean ±standard error measurement (n=3).

regard, none of the tested SWCNTs concentrations modified the NO production in presence (Fig. 5A) or absence (Fig. 5B) of ACh 10 textmu M. These data suggest that NO is not involved in the contraction effect induced by SWCNTs on the airway smooth muscle, and there may be other mediators implicated in their actions.

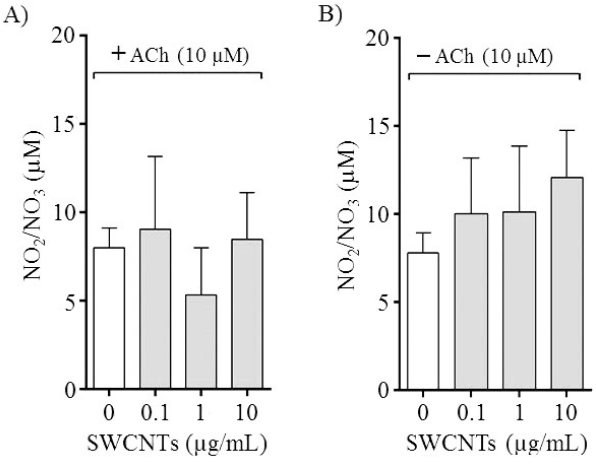

Figure 5: Effects of SWCNTs on NO production in the trachea rings. NO production, in absence and presence of SWCNTs (0.1, 1, and 10 µg/mL), was determined by the Griess method in presence (A) and absence (B) of ACh 10 µM. Values are represented as mean ±standard error measurement (n=3).

4 Discussion

The present work shows that SWCNTs induced a contractile effect in rat tracheal rings only in presence of ACh. The major finding was that SWNCTs stimulated a transient contraction similar to that induced by ACh, but in a minor magnitude. Furthermore, the SWCNTs-induced contraction was independent of both the SWCNTs concentration and the NO production. In this model, the null effect of SWCNTs in absence ACh pre-treatment suggest that the exposure and action of a contractile stimulator, such as ACh is necessary to activate intracellular signaling pathways on the airway smooth muscle to trigger signaling pathways capable to promote the contraction induced by SWCNTs. In concordance, our research group previously reported that other NMs, such as spherical 45 nm AgNPs, modified the ACh-induced contractile effect through the muscarinic receptor signaling and iNOS-mediated NO production in isolated rat tracheal rings (González et al., 2011). Thus, a specific airway hyperreactivity process induced by NMs is suggested.

Several studies have evaluated the effects of SWCNTs in biological systems. For instance, in a murine cremaster muscle model, SWNCTs with iron and gadolinium intercalated have opposite vasoactive effects depending on the aggregation rate. The aggregated SWNCTs induced dilation, while non-aggregated sonicated SWNCTs induced vasoconstriction (Frame et al., 2014). On the other hand, studies in aortic rings from Wistar rats showed that SWNCTs at a concentration range of 0.1-10 μg/mL induced an endothelium- and NO-independent vasodilation (Gutierrez-Hernandez et al., 2015). Our results suggest that the contractile effect is attributable to SWCNTs *per se* since EDS analysis revealed that SWCNTs lack of other intercalated metals. In contrast, Frame et al. (2014) employed SWCNTs having intercalated iron and gadolinium, which in turn could modify the mechanism of action and in consequence, the biological function of this kind of NM.

One important agent in the muscle tone regulation is NO (González et al., 2011), synthesized by NOS (Capettini et al., 2010; Sandoo et al., 2010). High NO levels induce peroxynitrite (Korhonen et al., 2005), leading to different cellular effects including cytotoxicity, cell proliferation, survival or apoptosis (De Boo et al., 2009; Feng et al., 2013).

In this work, the role of NO on the SWCNTs-induced airway contractile effect was evaluated. In this sense, the range of SWCNTs concentrations used modified the NO production regardless of the presence of Ach. These findings suggest that other mediators or a combination of them are released as a consequence of the interaction of ACh with the muscarinic receptor located in the airway smooth muscle cells (Kolahian and Gosens, 2012). However, the SWCNTs contractile effect may be related to a cytoskeleton disruption, and not necessarily involving NO (Zhang and Gunst, 2008). All data generated in the present study provide important information related to the inherent effects induced by SWCNTs as a promotor of the airway hyperreactivity that could act not only as a physiological marker in the development of respiratory pathogenesis, but also can provide important support in the expansion and development of new therapeutic alternatives. Further investigation is required to understand the effects and mechanisms of action induced by SWCNTs, as well as their fine interactions with the respiratory system structures.

Acknowledgement: This work was supported by Consejo Nacional de Ciencia y Tecnologia (CONACyT) through the fellowships of Salazar-García S (278347); Maldonado-Ortega D (633022), Ramirez-Lee MA (318669) and the grant C15-PIFI-06-02.02.

Conflict of interest statement: The authors declare no conflict of interest.

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