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Pulmonary Instillation of Multi-Walled Carbon Nanotubes Promotes Coronary Vasoconstriction and Exacerbates Injury in Isolated Hearts

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Abstract

The growing use of multi-walled carbon nanotubes (MWCNTs) across industry has increased human exposures. We tested the hypothesis that pulmonary instillation of MWCNT would exacerbate cardiac ischemia/reperfusion (I/R) injury. One day following intratracheal instillation of 1, 10, or 100 µg MWCNT in Sprague-Dawley rats, we used a Langendorff isolated heart model to examine cardiac I/R injury. In the 100 µg MWCNT group we report increased premature ventricular contractions at baseline and increased myocardial infarction. This was associated with increased endothelin-1 (ET-1) release and depression of coronary flow during early reperfusion. We also tested if isolated coronary vascular responses were affected by MWCNT instillation and found trends for enhanced coronary tone, which were dependent on ET-1, cyclooxygenase, thromboxane, and Rho-kinase. We conclude that instillation of MWCNT promoted cardiac injury by depressing coronary flow, invoking vasoconstrictive mechanisms involving ET-1, cyclooxygenase, thromboxane, and Rho-kinase.

Keywords

Nanomaterials; coronary resistance; premature ventricular contractions; endothelin-1; cyclooxygenase

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Declaration of Interest

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Introduction

Over the last two decades, structural, electrical, and thermal engineering has been enhanced with the development of multi-walled carbon nanotubes (MWCNTs). These materials are layers of graphite sheets rolled into concentrically arranged tubes that span microns in length but only nanometers in diameter (Pacurari et al. 2010). MWCNTs have extraordinary physicochemical properties and versatility that have made them promising materials to improve many modern goods (Miyagawa et al. 2005). Their durability and capacity for electrothermal conduction makes them attractive for use in compact electronic devices (Kis et al. 2006; Kis and Zettl 2008; Wang et al. 2011b), and MWCNTs are showing promise in many medical applications (Porter et al. 2010). With the increasing use of MWCNTs comes a greater need to understand the potential consequences of occupational inhalation and the resulting physiological responses (Gasser et al. 2012). In rodent models, pulmonary responses to MWCNT include persistent lung inflammation (Porter et al. 2010), pulmonary fibrosis (Mercer et al. 2011), and loss of lung function (Wang et al. 2011a). Cardiovascular endpoints are far less studied following pulmonary exposure to MWCNT, but a few studies have demonstrated that MWCNT exposure can increase microvascular endothelial permeability via oxidative stress (Pacurari et al. 2012), enhance serum lipid peroxidation (Reddy et al. 2011), and diminish serum antioxidant capacity (Reddy et al. 2011). It is reasonable to closely examine cardiovascular endpoints following MWCNT exposure because deleterious cardiovascular consequences have been shown to result from pulmonary exposure to other nanomaterials and particulates. Those consequences have been linked to oxidative stress (Cascio et al. 2007; Cozzi et al. 2006; LeBlanc et al. 2010), systemic inflammation (Wingard et al. 2010), cyclooxygenase (COX) activation (Knuckles et al. 2011), and/or altered endothelin signaling (Cherng et al. 2009; Cherng et al. 2011).

The isolated Langendorff heart model has been used to examine cardiovascular endpoints following nanoparticle exposure (Stampfl et al. 2011) and has also been used to assess changes in cardiac electrophysiology and ischemia/reperfusion (I/R) injury, in responses to various chemical compounds and pathological conditions (Bell et al. 2011; Skrzypiec-Spring et al. 2007). Endothelin-1 (ET-1) is a key autocrine/paracrine mediator during myocardial I/R due to its arrhythmogenic properties (Duru et al. 2001) and its vasoconstrictive effect, which impairs reperfusion flow (Pernow and Wang 1997). ET-1 is released during reperfusion in hearts subjected to I/R injury (Brunner et al. 1992) and both endogenous and exogenous ET-1 has been shown to contribute to infarct expansion during cardiac I/R (Ozdemir et al. 2006). Taken together with the release of ET-1 into the circulation during lung injury (Jesmin et al. 2011), ET-1 may be an important ligand to study following pulmonary exposure to MWCNT and myocardial infarction. Apart from ET-1, the RhoA/Rho-kinase (ROCK) pathway is associated with a number of pathological issues and is associated with sensitization of agonist-induced vasoconstriction (Somlyo and Somlyo 2000). Given that ROCK is associated with oxidative stress and hypertension following air pollution exposure (Sun et al. 2008), perhaps ROCK also contributes to cardiovascular complications following MWCNT exposure.

Collectively, the impacts on heart function during I/R, coronary responses to ET-1, and signaling cascades that recruit COX and ROCK have not been previously investigated

following MWCNT exposure, and was thus the purpose of this study. Here we used the Langendorff isolated heart technique to investigate cardiac I/R injury and wire myography to test vascular responsiveness in isolated coronary artery segments, both following pulmonary exposure to MWCNT. Specifically, we tested the hypothesis that intratracheal instillation of MWCNT would enhance coronary vasoconstriction via ET-1/COX/TXA₂- and ROCK-dependent mechanisms, ultimately increasing the vulnerability to cardiac I/R injury.

Methods

Animals

Male Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) at 12 weeks of age and given one to two weeks of acclimation before starting experimental protocols. They were housed in the Department of Comparative Medicine at East Carolina University with 12:12 hrs light-dark cycle and regulated temperature maintained at 23 ± 1°C. Rats were given access to standard laboratory chow and water *ad libitum*. All animal use complied with protocols approved by the Institutional Animal Care and Use Committee at East Carolina University.

Chemical Solutions

Vehicle instillate (10% surfactant/saline)—Solution by volume is 90% sterile saline (0.9 % NaCl) and 10% pulmonary surfactant (InfasurfTM, a gift from ONY, Inc., Amherst, NY).

Langendorff isolated heart perfusate—(mM) 118 NaCl, 24 NaHCO₃, 1.2 KH₂PO₄, 4.75 KCl, 1.2 MgSO₄, 2.0 CaCl₂, and 10 glucose (equilibrated with 95/5 % O₂/CO₂), heated to 37°C, as previously described (Brown et al. 2003; Frasier et al. 2011).

Physiological Saline Solution (PSS) for isolated coronary experiments—(mM) 140 NaCl, 5.0 KCl, 1.6 CaCl₂, 1.2 MgSO₄, 1.2 3-[N-morpholino]-propane sulfonic acid (MOPS), 5.6 d-glucose, and 0.02 EDTA (equilibrated with room air), with a pH of 7.4 when heated to 37°C.

Pharmacological agents for vascular studies—ET-1 (American Peptide Company, Inc., Sunnydale, CA) was dissolved in 0.1% acetic acid. FR139317 (R&D Systems, Inc., Minneapolis, MN) was dissolved in 100 mM DMSO. Indomethacin (Sigma-Aldrich, St. Louis, MO) was dissolved in 100% ethanol. DUP-697 (R&D Systems, Inc.) was dissolved in 100 mM DMSO. Ozagrel HCl (R&D Systems, Inc.) was dissolved in PSS. AA2414 (R&D Systems, Inc.) was dissolved in 100 mM DMSO. U46619 (R&D Systems, Inc.) was dissolved in 100% ethanol. HA-1077 (R&D Systems, Inc.) was dissolved in PSS. Serotonin (Sigma-Aldrich) was dissolved in PSS.

Multi-walled Carbon Nanotubes (MWCNT) and Exposure

The multi-walled carbon nanotubes (MWCNT) and suspensions used in this study have been physically characterized elsewhere (Wang et al. 2011a). One day prior to sacrifice, animals

were anesthetized with Isoflurane. A bolus of 1, 10, or 100 µg of MWCNT suspended in 200 µl of vehicle, or vehicle only (200 µl) was delivered into the opening of the trachea using a micro-pipette. MWCNT were provided by NanoTechLabs, Inc. (Yadkinville, NC).

Ischemia/Reperfusion (I/R) Injury

One day following MWCNT instillation, animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (85/15 mg/kg, respectively). Upon the absence of reflexes, hearts were excised and immediately mounted onto a modified Langendorff apparatus utilizing aortic retrograde perfusion under constant pressure (75 mmHg), without electrical pacing, as previously described (Frasier et al. 2011). After 5 minutes of stable baseline measurements hearts were challenged with 20 min global ischemia and then reperfused for 120 min. Hearts were then serially sectioned and incubated in a 1% TTC solution for the determination of infarct size, as described previously (Brown et al. 2003; Sloan et al. 2011). Ventricular arrhythmias were scored as described previously (Frasier et al. 2011) and premature ventricular contractions were counted during the stable baseline period.

ET-1 in Coronary Effluent

During I/R experiments the vena cava were occluded and coronary effluent was collected by cannulating the pulmonary artery. Effluent samples (4–5 ml) were collected from the vehicle and 100 µg MWCNT groups at baseline, at the onset of reperfusion (0 min), at 5 min, and at 10 min into reperfusion (samples collected for approximately 30–50 sec at each time-point). Effluent samples were snap-frozen in liquid nitrogen and stored at –80°C until analysis. An ET-1 enzyme immunoassay plate (ADI-900-020A, Enzo Life Sciences, Farmingdale, NY) was used to determine ET-1 levels in the coronary effluent samples, per the manufacturer's instructions. Optical densities were determined with a plate reader (Synergy HT, BioTek Instruments, Winooski, VT) using Gen5 software. The assay detection limit for conversion to ET-1 concentration occurred at optical densities of 0.098, corresponding to 0.1 pg/ml. Optical densities <0.098 were taken as 0 pg/ml. ET-1 concentrations were then normalized per gram of heart tissue (pg/ml/g of tissue).

Isolated coronary wire myography

Twenty four hours post-MWCNT instillation, hearts were excised from a set of anesthetized rats, separate from those used in the Langendorff studies, and placed in ice cold PSS. Segments of the left anterior descending coronary artery (LAD) were carefully isolated and mounted into the chambers of a multi-channel wire myograph system (DMT 610M, Ann Arbor, MI). Following 1 hr equilibration (oxygenated PSS with periodic washouts, heated to 37°C), LAD segments were stretched progressively and length-tension relationships were established. The law of Laplace was used to determine the optimum resting tension for each vessel segment (90% of the internal circumference established at tensions equivalent to 100 mmHg), as described by Halpern and Mulvany (Halpern and Mulvany 1977). After another 30 min equilibration period, tissue viability was assessed by a 10 min K⁺ depolarization using 109 mM K⁺PSS, which is PSS containing equal molar substitution of Na⁺ with K⁺. After washing out with fresh PSS and return to baseline tension, 1.0 µM serotonin was delivered to precontract the LAD segments, followed by 3.0 µM acetylcholine (5 minutes each) to test for endothelial-dependent relaxation.

ET-1 dose-responses

After PSS washouts and tension relaxation, LAD segments were subjected to cumulative dose-responses to ET-1 (0.1 nM - 1.0 μ M). Due to the robust nature of ET-1 responses, we conducted pair-wise measurements of ET-1 responses in separate LAD segments from the same heart, with and without pharmacological antagonist and inhibitors for ET-1 receptor types A and B (ET_AR and ET_BR), COX-1, COX-2, TXA₂ synthetase (TS), and TXA₂ receptor (TP). We used concentrations of the selective ET_AR antagonist FR139317 that were selective for ET_AR (10 nM) or a higher concentration that would also partially antagonize ET_BR (10 μ M), based on IC₅₀ values reported by the supplier (R&D Systems, Inc., Minneapolis, MN). Indomethacin (10 μ M) was used as a general COX inhibitor and 1 μ M DUP-697 was used to selectively inhibit COX-2. The TS inhibitor Ozagrel HCl has not been described previously in rat coronary tissue, so we utilized two concentrations (100 nM or 10 μ M) in order to identify an effective dose. The TP antagonist AA 2414 was used at a 1.0 μ M concentration. The concentrations for Indomethacin, DUP-697, and AA2414 were selected just above the IC₅₀ values reported by the supplier.

TXA₂ mimetic and ROCK inhibitor dose-responses

Another group of LAD segments was used to conduct dose responses to U46619, a TXA₂ mimetic (R&D Systems, Inc.) over the concentration range of 1.0 nM - 10 μ M. Dose responses to HA-1077, a ROCK inhibitor (R&D Systems, Inc.), were also assessed between 1.0 nM - 30 μ M after vessels had been stably precontracted with 1.0 μ M serotonin.

Statistics

All data are reported as mean $\pm$ SEM. Graphpad Prism software (version 5, LaJolla, CA) was used to conduct statistical analyses. A Student's t-test was performed to determine statistical significance against vehicle data for premature ventricular contractions, ending left ventricular pressure, infarct size, coronary flow, and ET-1 concentration in coronary effluent during reperfusion. For percent change in ET-1 from baseline at different time-points, a two-way analysis of variance (ANOVA) was performed with a Bonferroni post-test. Vascular response data was analyzed using a repeated measures two-way ANOVA (Ludbrook 1994). P values are either reported or denoted with * for $P < 0.05$ or ** for $P < 0.01$.

Results

Cardiac I/R Injury

The impact of MWCNT exposure on *ex vivo* myocardial ischemia/reperfusion (I/R) injury is presented in Figure 1. Cardiac injury, as assessed by arrhythmia and infarction, was enhanced in isolated hearts 24 hrs following intratracheal instillation of MWCNT. Representative ECG traces (5 sec), taken from the 5 min baseline recordings prior to ischemia, reveal noticeable differences in the number of PVCs between isolated hearts from vehicle instilled rats and hearts from rats instilled with any mass of MWCNT (Fig. 1A). The number of premature ventricular contractions (PVCs) in isolated rat hearts during the 5 minute baseline period were higher ($P < 0.05$) in the 100 μ g MWCNT group (63.5 ± 16.9)

than the vehicle (16.7 ± 3.3) and the $1 \mu\text{g}$ MWCNT groups (13.2 ± 4.1) (Fig. 1B). PVCs in isolated hearts from rats exposed to $10 \mu\text{g}$ MWCNT (37.1 ± 11.3) were also higher ($P < 0.05$) than the $1 \mu\text{g}$ MWCNT group (Fig. 1B). Following I/R, infarct sizes were larger ($P < 0.05$) in isolated hearts from rats instilled with $100 \mu\text{g}$ MWCNT ($68.3 \pm 7.0\%$ of the zone at risk) compared to hearts isolated from the vehicle group ($50.6 \pm 3.9\%$ of the zone at risk) (Fig. 1C). During reperfusion, the time until the first episode of ventricular tachycardia or fibrillation (VT/VF) was slightly decreased (not statistically different) in isolated hearts from rats instilled with $100 \mu\text{g}$ MWCNT (31.2 ± 14.3 sec) as compared to vehicle (66.4 ± 23.5 sec) (Fig. 1D). Additionally, cardiac arrhythmia scores generated for the 2 hr reperfusion period were similar, ranging from 4.2 ± 0.7 to 4.8 ± 0.4 in hearts isolated from vehicle instilled animals compared to those from the $100 \mu\text{g}$ MWCNT group, respectively.

Left Ventricular Pressures and Coronary Flow

Left ventricular developed pressure (LVDP) was lower ($P < 0.05$) in hearts isolated from rats instilled with $1 \mu\text{g}$ (9.8 ± 2.4 mmHg) or $10 \mu\text{g}$ (8.0 ± 1.5 mmHg) MWCNT compared to hearts from vehicle instilled rats (26.8 ± 5.7 mmHg) (Fig. 2A). The LVDP was not significantly lower in isolated hearts from the $100 \mu\text{g}$ MWCNT group (17.8 ± 6.5 mmHg) as compared to the vehicle group (Fig. 2A). No differences were observed in the maximum left ventricular pressure (Max LVP) in isolated hearts throughout reperfusion from rats instilled with vehicle or $100 \mu\text{g}$ MWCNT (Fig. 2B). The minimum LVP (Min LVP) was slightly elevated (not statistically different) during the first 30 min of reperfusion in isolated hearts from the $100 \mu\text{g}$ MWCNT group compared to the vehicle group (Fig. 2C). Coronary flow was depressed by $\sim 30\%$ ($P < 0.05$) during the first 30 min of reperfusion in isolated hearts from rats instilled with $100 \mu\text{g}$ MWCNT (143 ± 18 ml/g) compared to those from rats instilled with the vehicle (201.3 ± 20.8 ml/g) (Fig. 3A and 3B).

Endothelin-1 (ET-1) in Coronary Effluent

To test the hypothesis that the observed impairment of coronary flow involved endothelin-1 (ET-1), we measured ET-1 in coronary effluents collected from isolated rat hearts. Since ET-1 levels were not different during baseline measurements (data not shown), we show the percent change in ET-1 levels from baseline at different time-points during early reperfusion. There was marked elevation ($P < 0.01$) in ET-1 in effluents collected at the 10 min mark from the MWCNT group ($43.34 \pm 10.4\%$ increase) compared to the vehicle group ($-3.22 \pm 8.9\%$) (Fig. 4A). The mean ET-1 concentrations in coronary effluent during early reperfusion increased ($P < 0.05$) from 0.25 ± 0.2 pg/ml/g of heart tissue in the vehicle group, to 1.05 ± 0.3 pg/ml/g of heart tissue in the MWCNT group (Fig. 4B).

Endothelin Receptor-dependent Coronary Vascular Responses

Since coronary flow was depressed and ET-1 release was increased in isolated hearts from animals exposed to MWCNT, we tested to see if isolated segments of the left anterior descending coronary artery (LAD) from rats instilled with $100 \mu\text{g}$ MWCNT had augmented responses to ET-1. LAD segments isolated from the hearts of rats instilled with MWCNT 24 hrs earlier, showed trends for increased maximal stress (7.8 ± 1.2 mN/mm²) as compared to LAD segments isolated from the hearts of vehicle instilled rats (6.2 ± 0.5 mN/mm²) in response to cumulative concentrations of ET-1 (0.1 nM – 1.0 μM). To test if these responses

were dependent on endothelin A receptor (ET_AR) or endothelin B receptor (ET_BR) we incubated LAD segments with 10 nM FR 139317, an ET_AR antagonist. We found that at lower concentrations of ET-1 (0.1 – 10 nM) stress levels were similar between LAD isolated from the hearts of MWCNT exposed rats and vehicle exposed rats. However, at higher ET-1 concentrations (10 nM – 1.0 μ M) the stress generated by ET_AR-antagonized LAD from the MWCNT group increased to levels similar to those of unantagonized LAD isolated from the hearts of MWCNT instilled rats (Fig. 5A). The LADs were also incubated with a concentration of FR 139317 (10 μ M) that would also partially antagonize ET_BR. In LAD isolated from the hearts of MWCNT instilled rats, stress generation in the presence of 10 μ M FR 139317 was similar to that seen in unantagonized LAD from the vehicle group, at higher ET-1 concentrations (10 nM – 1.0 μ M) (Fig. 5B).

Cyclooxygenase (COX)-dependent Coronary Vascular Responses

Given that cyclooxygenase (COX) has been linked to altered vascular responses to ET-1 (Karaa et al. 2006; Xu et al. 2005), we incubated LAD segments isolated from the hearts of MWCNT or vehicle instilled rats with 10 μ M Indomethacin, a general COX inhibitor, for pair-wise comparison to uninhibited LAD segments. We found that Indomethacin-inhibited LAD segments from the MWCNT instilled rats had developed stress levels similar to that of inhibited and uninhibited LAD segments from vehicle instilled rats (Fig. 5C). To determine if the contribution of the enhanced constrictor response during ET-1 stimulation is from COX-2, we incubated LAD segments with 1 μ M DUP-697 and found an average 15% reduction in stress (compared to ~24% reduction with Indomethacin) for segments isolated from rats previously instilled with MWNCT (Fig. 5D). LAD segments from the hearts of vehicle-instilled rats showed no reduction in stress when inhibited with either Indomethacin or DUP-697.

Thromboxane (TXA₂)-dependent Coronary Vascular Responses

Activation of COX can result in the release of thromboxane (TXA₂), which can enhance vasoconstriction. To determine the contribution of TXA₂ in the enhanced coronary tone, we incubated LAD segments with Ozagrel-HCl, a TXA₂ synthetase (TS) inhibitor, or AA 2414, a TXA₂ receptor (TP) antagonist, for pair-wise comparison to uninhibited LAD segments. LAD segments isolated from MWCNT instilled rats and inhibited with 100 nM Ozagrel-HCl demonstrated no reduction in the magnitude of developed stress (8.2 ± 2.1 mN/mm² vs. 7.8 ± 1.2 mN/mm² in uninhibited), but inhibition with 10 μ M Ozagrel-HCl reduced the ET-1 constrictor response to 5.9 ± 0.2 mN/mm², similar to the stress level achieved in LAD isolated from rats instilled with the vehicle (6.2 ± 0.5 mN/mm²) (Fig. 6A). Isolated LAD segments from MWCNT-instilled rats incubated with 1 μ M AA 2414 also had a 27 % reduction in ET-1 stress generation to as low as 5.7 ± 0.4 mN/mm² (Fig. 6B). AA 2414 had little impact in LAD coronary arteries from vehicle instilled rats. Given the implication for TXA₂ action during ET-1 dose-responses, we also sought to determine if previous MWCNT instillation resulted in differences in vascular responses to TXA₂. Interestingly, LAD mean maximal stress to U46619, a TXA₂ mimetic, were slightly lower in the MWCNT group (2.5 ± 0.4 mN/mm²) than in the vehicle group (4.1 ± 0.8 mN/mm²) (Fig. 6C).

Rho-kinase (ROCK)-dependent Coronary Vascular Responses

Enhanced coronary vasoconstriction has also been shown to be a result of Rho-kinase (ROCK) activation (Sato et al. 2011). We examined ROCK mediated diminution of vascular tone by first precontracting LAD segments with serotonin (5-HT), and then applying cumulative doses of HA-1077, a ROCK inhibitor. We selected 5-HT to precontract vessels after dose-response data (Supplemental Table I) and stress-response data for 5-HT (Supplemental Fig. 1A) revealed no differences in maximal stress between MWCNT and vehicle instilled groups. LAD from MWCNT instilled rats showed trends for higher IC_{50} values for HA-1077 ($\text{Log M } IC_{50} = -5.7 \pm 0.5$) than those from the vehicle group ($\text{Log M } EC_{50} = -6.4 \pm 0.5$) (Fig. 6D). Dose-response comparisons of 2-Cl-Adenosine, acetylcholine (ACh) and sodium nitroprusside (SNP) were also examined in LAD from MWCNT or vehicle instilled rats in order to exclude impairment of endothelial-dependent and -independent relaxation responses (see supplemental Table I and Supplemental Fig. 1B, C, and D).

Discussion

This is the first study showing that pulmonary instillation of MWCNTs 24 hrs prior to induction of cardiac ischemic injury resulted in deleterious *ex vivo* cardiovascular endpoints. Herein, we demonstrated that pulmonary instillation of MWCNTs increased myocardial infarction following I/R injury in isolated rat hearts, which was associated with increased PVCs prior to the ischemic bout and depression of coronary flow during early reperfusion. Further, MWCNT instillation augmented ET-1 release from the isolated hearts during early reperfusion and myography data obtained from isolated LAD suggests that contributing factors to the depression of coronary flow may have been enhanced coronary vasoconstriction via ET-1, COX, elements of the TXA_2 axis and ROCK. These findings are consistent with cardiovascular complications associated with particulate matter and air pollution exposure, which are dependent on decreasing size, increasing number per mass and increasing surface area per mass of the particulate (Brook et al. 2010; Hussain et al. 2009; Legramante et al. 2009). MWCNT have similar physicochemical properties, including high aspect ratios, high particle numbers per mass, and high surface area to mass ratios, which has raised concerns about their ability to initiate cardiovascular complications following pulmonary exposure. However, until now it has not been clear whether pulmonary exposure to MWCNT would generate biological responses capable of altering intrinsic physiological processes of the heart and coronary vasculature, and if so, which mechanisms, functions, and signaling cascades would be altered.

The MWCNTs utilized in this investigation were physically characterized in a previous study showing that pulmonary exposure generated moderate pulmonary immune cell infiltration, collagen deposition, granuloma formation, and impairment of pulmonary function (Wang et al. 2011a). We used three bolus mass concentrations of MWCNTs delivered by intratracheal instillation in rats for the intention of establishing whether MWCNTs could exert effects beyond the lung, similar to what is known regarding ambient particulate matter. While this approach does not mimic real-world exposure conditions or deposition patterns in the lung that would occur via inhalation, it provides a model to test

whether these MWCNTs may influence the cardiovascular system. Future work should focus on similar endpoints following an inhalation exposure, which is more physiologically relevant and would provide critical data needed to develop a risk assessment paradigm.

We used the isolated Langendorff rat heart model of I/R injury to examine cardiac endpoints that were dependent on changes in intrinsic characteristics of the heart and local signaling cascades, rather than MWCNT-induced changes in autonomic outflow and circulating humoral factors at the time of I/R. The isolated heart model has been described elsewhere as an appropriate system for investigating the cardiovascular consequences of direct toxicology (Skrzypiec-Spring et al. 2007) or nanoparticle exposure, via delivery of nanoparticles within the isolated heart perfusate (Stampfl et al. 2011). Our study demonstrates that the isolated heart model is also useful in assessing cardiovascular endpoints following pulmonary exposure to nanomaterials. The model provided simultaneous measurements of electrical activity/arrhythmogenesis, cardiac function/contractility, and coronary flow throughout the I/R protocol (Bell et al. 2011). We were able to compare these types of data to subsequent myocardial infarction and loss of LVDP that occurred by the end of I/R.

The first novel finding in this study is that pulmonary exposure to MWCNTs resulted in cardiac arrhythmogenic injury. Hearts isolated from animals exposed to 10 or 100 µg of MWCNT had more PVCs prior to the ischemic bout than those hearts taken from animals instilled with vehicle or 1 µg MWCNT. These data are consistent with other studies that have noted increased arrhythmogenesis and PVCs following pulmonary exposure to particulate matter (He et al. 2011; Kang et al. 2002; Kim et al. 2012). Contributing factors like MWCNT-dependent effects on autonomic outflow and/or circulating humoral factors are greatly diminished in the isolated heart model, but neurotransmitter release from the remaining nerve endings in the heart could remain influential (Stampfl et al. 2011). One possible explanation for increased PVCs at baseline is a possible increase in circulating oxidants in the hours following MWCNT exposure, which could prime the arrhythmogenic responses seen during the baseline ECG tracings. There is supporting evidence that MWCNTs have been shown to increase oxidative burdens in the vascular compartment (Reddy et al. 2011) and increased PVCs associated with particulate matter exposure have been linked to oxidative stress (Kim et al. 2012). Furthermore, it is possible that the basal PVC generation in isolated hearts could be a product of oxidant stress in myocardial tissue following pulmonary exposure to MWCNTs. Within the myocardium, oxidative stress is known to increase the propensity for arrhythmia (Brown and O'Rourke 2010), increase spontaneous calcium release from the sarcoplasmic reticulum (Belevych et al. 2009), and introduce heterogeneity into myocardial action potentials (Brown and O'Rourke 2010), all of which are known to increase the proclivity for electrical dysfunction. Interestingly, during reperfusion, arrhythmia scores were only slightly higher in hearts from MWCNT exposed rats compared to those from the vehicle group, but trends for decreased time until the first VT/VF and increased time spent in VT/VF remained evident. The 20 min global ischemia challenge and subsequent reperfusion period likely pushed all the hearts in this study into such a highly arrhythmogenic state that MWCNT exposure was unable to worsen arrhythmogenesis during reperfusion. In either case, we propose that increased arrhythmogenesis at baseline following pulmonary exposure to MWCNT could possibly

serve as an indicator of cardiac vulnerability to I/R, and further investigation of this observation should be warranted.

The second novel finding in this study is the exacerbation of myocardial infarction following I/R in isolated rat hearts. Given that increased levels of endogenous ET-1 has been linked to increasing myocardial infarction following I/R (Ozdemir et al. 2006), it is possible that the increased ET-1 in the effluents of isolated hearts seen during reperfusion in the 100 μ g MWCNT group contributed to the infarct expansion. It is also tempting to speculate that the exacerbated size of myocardial infarction in isolated hearts taken from rats instilled with 100 μ g MWCNT may have prevented worsening of the arrhythmogenic state of these hearts during reperfusion. It has been demonstrated that exacerbated myocardial infarction potentially insulates the tissue against injury currents, which are abnormal electrical wave front propagations (Luca 1979), and could also explain the similarity in arrhythmia scores during reperfusion between isolated hearts from rats instilled with vehicle or 100 μ g MWCNT. Clearly, further research will be needed in order to substantiate such a claim, but given the complexity and multifaceted aspects of cardiac I/R injury, such research would have multidisciplinary benefits outside of MWCNT-associated cardiotoxicity. MWCNT instillation also appeared to have some effect on post-I/R cardiac contractility. In particular, those hearts isolated from rats instilled with lower mass concentration (1 or 10 μ g) of MWCNTs had lower LVDP by the end of reperfusion than those hearts isolated from the vehicle instilled rats. LVDP is the difference between Min LVP and Max LVP and describes the force generating capacity of the left ventricle, and is thus commonly used as a measure of cardiac function. Such a reduction in LVDP following I/R could result from cardiac stunning, arrhythmia, infarction, or any combination thereof (Brown and O'Rourke 2010; Zweier and Talukder 2006). Interestingly, hearts isolated from rats instilled with the highest concentration (100 μ g) of MWCNTs did not demonstrate the same magnitude of LVDP reduction by the end of reperfusion that was seen with lower MWCNT doses. TXA₂ is linked to cardiac I/R and increased arrhythmias (Coker et al. 1981), and TXA₂ has been shown to elevate intracellular Ca²⁺ in cardiac myocytes (Dogan et al. 1997). Given that our study shows MWCNT instillation promoted increased ET-1 release from isolated hearts during early reperfusion, and our vascular data that suggests coronary tone is enhanced through TXA₂ release in response to ET-1, perhaps MWCNT activation of the ET-1/TXA₂ axis enhances contractility of cardiac myocytes, preserving some ventricular function in the face of expanding injury.

The third novel finding in this study was that coronary reperfusion flow was depressed in hearts from rats instilled with 100 μ g MWCNTs when compared to the vehicle group. Adequate coronary reflow is important due to the necessity of oxygen and nutrient delivery to the ischemic region in order to maintain cardiac contractility and myocardial viability, but these benefits are contrasted by increased oxygen free radical production in response to coronary reperfusion (Cadenas et al. 2010). Enhanced coronary vasoconstriction, and thus increased vascular resistance, is a central mechanism responsible for decreases in coronary blood flow, ultimately contributing to cardiac arrhythmogenesis and infarction during early post-ischemic reperfusion (Li et al. 2012). In this study, the modified Langendorff heart model delivers perfusate under constant pressure. Therefore, the physical relationship of $R = P/Q$ were considered, where R stands for change in vascular resistance in the face of

constant perfusion pressure (P) and an inverse change in the rate of perfusate flow (Q). The depression in flow during early post-ischemic reperfusion of isolated hearts taken from rats previously instilled with 100 μ g MWCNTs suggested that vascular resistance was elevated. This may have been partially explained by increases in Min LVP and increased ET-1 release.

The elevated ET-1 levels in the coronary effluent could have also contributed to the depression of coronary flow (as depicted in Fig. 7), since ET-1 is a potent vasoconstrictor (Callera et al. 2007). Increased ET-1 levels in coronary effluents during reperfusion in hearts from the 100 μ g MWCNT group are also consistent with the increased release of endothelins previously documented following particulate matter exposure and myocardial infarction (Kang et al. 2002). Furthermore, ET-1 release has been demonstrated in isolated Langendorff rat hearts subjected to I/R at a level and pattern similar to that of our vehicle group (Brunner et al. 1992). This suggests the burst of ET-1 in the coronary effluents collected from the isolated hearts of MWCNT exposed rats, 10 min into reperfusion, may in fact be a MWCNT effect. Cardiac responses to arrhythmogenic autocrine/paracrine agents like ET-1 could explain the increases in PVCs (Duru et al. 2001; Kang et al. 2002), though our data shows that ET-1 levels in preischemic coronary effluent samples were not different between groups (data not shown). This brings into question the possibility that MWCNT exposure may perhaps modulate the response to ET-1 in cardiac myocytes and nodal tissue. Cardiac infarct size has also been shown to relate to the amount of ET-1 released (Ozdemir et al. 2006), indicating that elevated ET-1 levels during reperfusion in isolated hearts from rats exposed to 100 μ g MWCNT may have also contributed to exacerbated myocardial infarction. The role of ET-1 during myocardial I/R has been extensively reviewed (Pernow and Wang 1997), but the impact of MWCNT exposure on cardiac and coronary responses to ET-1 have not been previously identified. Based on this study, a more in-depth assessment of myocardial and vascular responses to ET-1 represents a logical focus point for future research following MWCNT exposure.

Since coronary flow was depressed during early post-ischemic reperfusion in isolated hearts from the 100 μ g MWCNT group, we tested the hypothesis that ET-1 vasoconstriction in isolated LAD segments taken from rats exposed to 100 μ g MWCNT would be enhanced in a COX/TXA₂-dependent manner. ET-1 is a potent vasoconstrictor released from endothelial cells, though not exclusively, to influence local vascular tone (Callera et al. 2007). On vascular smooth muscle cells, ET-1 acts on ET_AR or ET_BR, which are both coupled to G_q proteins. On vascular endothelial cells, ET-1 can act on ET_BR coupled to G_i proteins (Desjardins et al. 2005). ET_AR activation generally produces strong and prolonged vasoconstriction through G_q activation of phospholipase C, mobilization of inositol triphosphate and diacylglycerol from the lipid bilayer, which in turn activates Ca²⁺ release from the sarcoplasmic reticulum and extracellular Ca²⁺ entry via voltage operated channels, producing vascular smooth muscle contraction (Kohan et al. 2011). ET_BR activation on endothelial cells produces vasodilation by stimulating release of NO, which results in vascular smooth muscle relaxation (Kohan et al. 2011). Oxidative stress has been shown to alter vascular responses to ET-1 through activation of the COX pathway, resulting in release of thromboxane (TXA₂) from vascular endothelial cells, and thus exaggerating vasoconstriction (Karaa et al. 2006; Xu et al. 2005). There is evidence that suggests this may

be a result of endothelin B receptor (ET_BR) activation (Miller and Zhang 2009; Plante et al. 2002).

Under inflammatory conditions ET-1 has been linked to increasing arachidonic acid metabolism through activation of the COX pathway, resulting in release of TXA₂, and thus exaggerated vasoconstriction (Karaa et al. 2006; Xu et al. 2005). Other evidence suggests induction of COX-2 specifically mediates TXA₂ release in response to ET-1 (Qi et al. 2007). Our data suggest that COX-2 and TXA₂ play a role in coronary responses to ET-1, 24 hrs following MWCNT exposure, as depicted in Fig. 7. As mentioned previously TXA₂ has also been shown to increase intracellular Ca²⁺ in cardiac myocytes (Dogan et al. 1997). Given that Ca²⁺ overload is prominently involved in the development of necrotic/apoptotic cell death during early reperfusion (Halestrap 2009; Murphy and Steenbergen 2008) it seems likely that TXA₂ could have simultaneously contributed to both the exacerbated infarct size as well as the coronary responses in the 100 µg MWCNT group. We also showed that LAD stress-responses to U46619, a TXA₂ mimetic, generated less stress in the MWCNT group compared to vehicle, possibly indicating TXA₂ receptor (TP) desensitization in LAD isolated from rats exposed to 100 µg MWCNTs. Such an agonist-induced desensitization of TP is thought to occur through protein kinase C activation and TP phosphorylation by G-protein receptor kinases (Flannery and Spurney 2002). Therefore, if COX is basally active following MWCNT exposure, continued TXA₂ release could result in TP desensitization. In either case, the involvement of the ET-1/COX/TXA₂ signaling axis may be a plausible mechanism to explain the depression of coronary flow during early reperfusion seen in the Langendorff isolated heart experiments, following MWCNT exposure (Fig. 7). The constrictor responses of the isolated coronary vessels with ET_AR antagonism maintained higher stress levels than those also coupled with ET_BR antagonism. Given that ET_BR activation has been linked to COX activity and TXA₂ release in other vascular beds (Miller and Zhang 2009; Plante et al. 2002), perhaps our findings indicate a similar mechanism in coronary arteries following MWCNT exposure.

The Rho-A/ROCK pathway is often implicated in the sensitization of agonist-induced vasoconstriction and is a well-documented mechanism that drives a variety of pathological issues (Somlyo and Somlyo 2000). ROCK is a serine/threonine protein kinase that can modulate Ca²⁺ sensitivity of smooth muscle myofilaments and enhance vascular tone (Nunes et al. 2010). Further, ROCK has been shown to increase vascular tone and potentiate hypertension following exposure to air pollution (Sun et al. 2008). Our data suggest that ROCK may also contribute to stress development in coronary arteries following MWCNT instillation, which we have also incorporated into Fig. 7. The rightward shift in the dose-response curve for HA-1077, a ROCK inhibitor, in LAD segments isolated from rats instilled with 100 µg MWCNTs indicates that agonist-induced vasoconstriction may be modified in a ROCK-dependent manner, which can cause enhancement of vascular tone via agonist-induced Ca²⁺ sensitization (Nunes et al. 2010; Randriamboavonjy et al. 2003; Somlyo and Somlyo 2000). When taken together with the indications that ET-1 responses may be augmented via the COX/TXA₂ axis, enhanced vascular tone could very well have contributed to the depression of coronary flow and ultimately to the increased propensity to injury seen in our isolated hearts.

Conclusions

We have demonstrated using an isolated Sprague-Dawley rat heart Langendorff model that 24 hrs following pulmonary instillation of MWCNT *in vivo*, intrinsic changes have occurred in the heart and coronary vasculature that promote *ex vivo* cardiac I/R injury. These include basal arrhythmogenesis, increased reperfusion ET-1 release, and depression of coronary flow during reperfusion, all of which likely contribute to post-I/R myocardial infarct expansion. We further suggest that activation of COX/TXA₂ axis through stimulation with ET-1 establishes a sensitized constrictor state of coronary blood vessels. Furthermore, ROCK may also contribute to the enhanced vascular tone in coronary arteries following MWCNT instillation. Taken together, pulmonary instillation of MWCNT can drive adverse systemic responses outside of the lungs, which will likely impact the heart and coronary vasculature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Pervious pulmonary instillation of MWCNT increases basal arrhythmogenesis and increases infarction following ischemia/reperfusion (I/R) in isolated rat hearts. 24 hrs following intratracheal instillation of MWCNT or vehicle in rats, hearts were isolated, perfused, and subjected to 20 minutes of global ischemia followed by 120 minutes of reperfusion. (A) Five sec representative ECG tracings from the baseline period prior to ischemia, which shows the frequency of premature ventricular contractions (PVCs) in hearts from MWCNT instilled animals. (B) Quantification of PVCs during baseline (prior to ischemia). (C) Infarct sizes at the end of I/R protocols, reported as a percent of the zone at risk (ZAR). (D) Time into reperfusion before the first episode of ventricular tachycardia or fibrillation (VT/VF) occurred. N=6–10. * P < 0.05 vs. vehicle unless otherwise noted.

Figure 2.

Pulmonary instillation of MWCNT impaired left ventricular developed pressure (LVDP) by the end of reperfusion in isolated hearts subjected to I/R. (A) Mean LVDP at the end of the I/R protocols. (B) Tracing of the maximum left ventricular pressure (Max LVP) throughout the cardiac I/R experiments. (C) Tracing of the minimum left ventricular pressure (Min LVP) throughout the isolated cardiac I/R experiments. N=6–10. * $P < 0.05$ vs. vehicle.

Figure 3.

Pulmonary instillation of MWCNT resulted in depression of coronary flow during early post-ischemic reperfusion in isolated rat hearts. (A) Tracing of the minute perfusion volume, showing approximately a 30% reduction in flow during the first 30 minutes of reperfusion. (B) Determined by area under the curve analysis of panel A during first 30 min of reperfusion. N=6–10. * $P < 0.05$ vs. vehicle.

Figure 4.

Pulmonary instillation of MWCNT (100 μ g) increased endothelin-1 (ET-1) release into coronary effluent during early reperfusion in isolated rat hearts subjected to I/R. Coronary effluent samples were collected at the start of stable baseline recordings, before the onset of cardiac I/R, and then again during reperfusion at 0 min, 5, and 10 minutes into reperfusion. (A) Percent change in baseline (preischemic) optical density values reported from an ET-1 enzyme immunoassay plate (Enzo Life Sciences). (B) ET-1 concentrations during reperfusion (0–15 min) were extrapolated from the standard curve. N=4. **P < 0.01 vs. vehicle. *P < 0.05 vs. vehicle.

Figure 5.

Pulmonary instillation of MWCNT showed trends for increased ET-1-dependent coronary tone via endothelin B receptors (ET_B R) and cyclooxygenase-2 (COX-2) in segments of the left anterior descending coronary artery (LAD) that were isolated from rats instilled with 100 μ g MWCNT. Twenty-four hrs after intratracheal instillation of vehicle or 100 μ g MWCNT, segments of the LAD were isolated for wire myography analysis of ET-1 response curves (0.1 nM – 1.0 μ M). (A) ET-1 stress-response curves from isolated LAD incubated with and without 10 nM FR 139317, an endothelin A receptor (ET_A R) antagonist, from the hearts of 100 μ g MWCNT exposed rats. (B) ET-1 stress-response curves from isolated LAD incubated with and without 10 μ M FR 139317, which antagonizes ET_A R and partially antagonizes ET_B R, from rats exposed to 100 μ g MWCNT. (C) ET-1 stress-response curves from isolated LAD incubated with and without 10 μ M Indomethacin (Indo), a general COX inhibitor, from rats instilled with 100 μ g MWCNT or vehicle. (D) ET-1 stress-response curves from isolated LAD incubated with and without 1.0 μ M DUP-697, a COX-2 selective inhibitor, from rats instilled with 100 μ g MWCNT or vehicle. N=4–8.

Figure 6.

Pulmonary instillation with MWCNT showed trends for increased maximal coronary isometric stress responses to ET-1 via thromboxane (TXA₂) signaling and for increased Rho-kinase (ROCK)-dependent coronary tone in LAD segments isolated from rats instilled with 100 μ g MWCNT or vehicle. (A) ET-1 stress-response curves with different doses of Ozagrel HCl, a TXA₂ synthetase inhibitor, in LAD from MWCNT instilled rats. (B) ET-1 stress-response curves with and without 1.0 μ M AA 2414, a TXA₂ receptor antagonist, in isolated LAD taken from the hearts of vehicle and 100 μ g MWCNT instilled rats. (C) Stress-response curves for U46619, a TXA₂ mimetic, in isolated LAD from vehicle and MWCNT treated rats. (D) Dose-response curve for HA-1077, a ROCK inhibitor, in isolated LAD from vehicle and 100 μ g MWCNT instilled rats, following precontraction with 1.0 μ M serotonin. N=4–8.

Figure 7.

Pulmonary instillation of MWCNT appears to enhance coronary tone through increased ET-1 release from the heart during I/R. We also uncovered trends for activation of the COX/ TXA_2 axis in response to ET-1 and potential contributions of ROCK to enhance coronary tone.