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Airway Exposure to Modified Multi-walled Carbon Nanotubes Perturbs Cardiovascular Adenosinergic Signaling in Mice

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Abstract

The broad list of commercial applications for multi-walled carbon nanotubes (MWCNT) can be further expanded with the addition of various surface chemistry modifications. For example, standard commercial grade MWCNT (C-grade) can be carboxylated (COOH) or nitrogen-doped (N-doped) to suite specific utilities. We previously reported dose-dependent expansions of cardiac ischemia/reperfusion (I/R) injury, 24 h after intratracheal instillation of C-grade, COOH, or N-doped MWCNT in mice. Here, we have tested the hypothesis that airway exposure to MWCNT perturbs cardiovascular adenosinergic signaling, which could contribute to exacerbation of cardiac I/R injury. 100 µL of Vehicle or identical suspension volumes containing 100 µg of C-grade, COOH, or N-doped MWCNT were instilled into the trachea of CD-1 ICR mice. 1 day later, we measured cyclic adenosine monophosphate (cAMP) concentrations in cardiac tissue and evaluated arterial adenosinergic smooth muscle signaling mechanisms related to nitric oxide synthase (NOS) and cyclooxygenase (COX) in isolated aortic tissue. We also verified cardiac I/R injury expansion and examined both lung histology and bronchoalveolar lavage fluid cellularity in MWCNT exposed mice. Myocardial cAMP concentrations were reduced ($p < 0.05$) in the C-grade group by 17.4% and N-doped group by 13.7% compared to the Vehicle group. Curve fits to aortic ring 2-Cl-Adenosine concentration responses were significantly greater in the MWCNT groups vs. the Vehicle group. Aortic constrictor responses were more pronounced with NOS inhibition and were abolished with COX inhibition. These findings indicate that addition of functional chemical moieties on the surface of MWCNT may alter the biological responses to exposure by influencing cardiovascular adenosinergic signaling and promoting cardiac injury.

Keywords Multi-walled carbon nanotubes · Cardiac ischemia/reperfusion injury · Adenosine · cAMP

Introduction

Multi-walled carbon nanotubes (MWCNT) have dynamic applicability across industries and present potential for occupational exposure by inhalation during production and handling [1]. The physicochemical properties of MWCNT have been shown to dictate biological responses to airway exposures [2], which raises questions regarding what MWCNT modifications can contribute to these observations. Standard commercial grade MWCNT (C-grade) have high chemical and thermal stability. In order to increase MWCNT electrothermal conduction, the surface of MWCNT can be doped with nitrogen moieties (N-doped) [3]. MWCNT can be functionalized with carboxylic acid groups (COOH) to enhance solubility [4]. We have previously reported that intratracheal instillation of C-grade, COOH, and N-doped MWCNT caused a dose-dependent and time-dependent

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expansion of cardiac ischemia/reperfusion (I/R) injury in C57BL/6 mice as early as 24 h after exposure [5]. However, it remains unclear what physiological mechanisms contributed to the expansion of cardiac I/R.

Adenosinergic signaling regulates an array of cardiovascular functions [6]. Reduced concentrations of 3′,5′-cyclic adenosine monophosphate (cAMP) have been implicated in cardiovascular pathologies [7, 8] and increased cAMP concentration provides cardioprotection against I/R injury [9, 10]. Generally, this protection is thought to occur because cAMP activates protein kinase A signaling cascades and influences mitochondrial permeability transition pore, ROS generation, and various channels and cell types in cardiovascular tissues [11, 12]. Cardiac cAMP may also be linked to adenosinergic dysfunction because cAMP is converted to adenosine via the cAMP-adenosine pathway [13]. Adenosine itself is an important autocrine-paracrine signaling agent in both heart [14] and vascular tissue [15], and is usually regarded as tissue protective in acute settings [16, 17]. The local action of adenosine allows for the titration of local blood flow during changes in autonomic tone. For example, adenosine has been documented to attenuate α-adrenergic vascular tone [18]. Adenosine regulates vascular tone via activation of both nitric oxide (NO) [19] and cyclooxygenase (COX) signaling [20], and is thought to be released during ischemia [21, 22]. In contrast, adenosine has also been reported to augment aortic constrictor responses mediated through selective adenosine receptors and endothelial cell signaling [23–25]. Thus, altered vascular responsiveness to adenosine associated with MWCNT exposure could negatively impact cardiac I/R injury.

The purpose of this study was to test the influence of modified MWCNT exposure on cardiovascular adenosinergic systems. We hypothesized that pulmonary exposure to MWCNT would decrease cAMP concentrations in the heart and alter adenosinergic vascular responses through changes in NO and COX signaling. We selected the MWCNT dose based on similar levels of pulmonary injury between MWCNT types, 1 day after exposure in C57BL/6 mice in our previous study [5]. Here, we have assessed pulmonary inflammation, verified cardiac I/R injury, measured heart and lung cAMP concentrations, and interrogated adenosinergic responsiveness in vascular tissue 1 day after intratracheal instillation of MWCNT in CD-1 ICR mice.

Materials and Methods

Animals

Male CD-1 ICR mice were purchased from Charles River (Raleigh NC, USA) at 4–6 weeks of age. After arrival at East Carolina University's Department of Comparative Medicine, the mice were allowed to acclimate for at least 1 week prior to experimental procedures. Mice were housed in temperature controlled rooms (23 ± 1 °C) with 12-h light/dark cycles and provided access to standard laboratory chow and fresh water ad libitum. The use of mice in this study complied with protocols approved by the Institutional Animal Care and Use Committee at East Carolina University (Greenville, NC, USA).

MWCNT Characterization

In this study, unmodified commercial grade (C-grade), acid-washed carboxylated (COOH), and nitrogen-doped (N-doped) MWCNT were examined. All MWCNT were derived by chemical vapor-deposition and kindly provided by NanoTecLabs, Yadkinville NC, USA. The MWCNT used in this study have been characterized previously in both dry form and in suspension and are free of endotoxin contamination [5].

MWCNT Instillation

Experimental suspensions of C-grade, COOH, and N-doped MWCNT were prepared at 1 mg/mL in sterile10% pulmonary surfactant/saline using Infasurf™ (ONY, Inc., Amherst NY, USA). Infasurf™ contains 35 mg of bovine phospholipids, including 16 mg disaturated phosphatidylcholine; 0.7 mg bovine proteins, including 0.44 mg hydrophobic surfactant-associated protein C and 0.26 mg hydrophobic surfactant-associated protein B (http://www.infasurf.com/about-infasurf/#composition). 24 h prior to cardiopulmonary assessments, the suspensions were sonicated in a bath sonicator for 20 min to generate approximately 10,000 J. Immediately after sonicating the MWCNT, mice were anesthetized with Isoflurane and then intratracheally instilled with 100 µL suspensions containing 0 (Vehicle) or 100 µg of either C-grade, COOH, or N-doped MWCNT.

Heart, Lung and Aorta Collection

In the cohort of mice not used for ischemia/reperfusion experiments, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (90/10 mg/kg, respectively) and exsanguinated by cutting the inferior vena cava. Hearts and thoracic aortas were resected and placed on ice. Aortas were placed in cold physiological saline solution (PSS) containing (in mM) 140 NaCl, 5.0 KCl, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 1.2 3-[*N*-morpholino]-propane sulfonic acid (MOPS), 5.6 D-glucose, and 0.02 EDTA for myographic assessments. Hearts were snap frozen in liquid nitrogen and stored at − 80 °C until analyzed. The intact lung tissue was either used for bronchoalveolar lavage studies, histological fixation, or

was collected for tissue analysis, in which case it was snap frozen in liquid nitrogen and stored at − 80 °C.

Bronchoalveolar Lavage (BAL) and Lung Histology

Right lungs were lavaged in situ four times with 26.25 mL/kg of cold Hanks balanced salt solution (HBSS) as previously described [26]. Each of the four individual lavage samples were collected separately and centrifuged at 4 °C for 10 min at 1000 × *g*. The supernatant from the first lavage sample was used for protein quantification. Each cell pellet was combined and re-suspended in 1 mL of HBSS to derive total cell counts using a Cellometer® Auto X4 (Nexcelcom Biosciences, LLC., Lawrence, MA, USA). A volume of cell suspension containing approximately 20,000 cells was centrifuged onto glass slides using a Cytospin IV (Shandon Scientific Ltd., Cheshire, UK) and stained with three-step hematology stain (Richard Allan Scientific, Kalamazoo, MI, USA). Differential cell counts were derived by microscopically evaluating 300 cells per slide based on hematological staining and morphology. For each cell type per slide, the percentage of 300 cells was calculated and multiplied by the total cell count for data reporting. Unlavaged left lungs were inflated with 10% neutral buffered formalin at 25 cm of H_2O and incubated at room temperature for 24–72 h. Fixed lung tissue was cut, processed, embedded in paraffin, sectioned 5 µm thick, mounted on slides, and stained with hematoxylin and eosin (H&E).

Ischemia/Reperfusion (I/R) Injury

Twenty-four hours after instillation, one cohort of mice was subjected to an experimental cardiac I/R protocol as previously described [5]. In short, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (90/10 mg/kg, respectively), then intubated, and mechanically ventilated. A midline parasternal thoracotomy was conducted to access the left anterior descending coronary artery (LAD), which was then ligated with a reversible tourniquet to induce ischemia in the zone of tissue distal to the occlusion (i.e. zone at risk; ZAR). After 20 min, the ligature was reversed and the ZAR was allowed to reperfuse for 2 h, followed by exsanguination of the inferior vena cava. In order to demarcate the ZAR, the LAD was religated, the aorta was cannulated, and Evans blue dye was perfused in a retrograde fashion to stain myocardial tissue not in the ZAR. The left ventricle was then excised, serially sectioned, and incubated in a 1% triphenyltetrazolium chloride solution to demarcate the infarcted tissue within the ZAR. Triphenyltetrazolium is enzymatically converted into a brick red substance in viable tissue. Infarcted tissue remains pale. Differential staining was analyzed by computer planimetry and infarction is reported as percent of the ZAR.

Adenosine 3′,5′-Cyclic Monophosphate (cAMP) ELISA

An enzyme-linked immunosorbent assay (ELISA) for cAMP (cat# ADI-900-163, Enzo Life Sciences, Farmingdale, NY, USA) was used to determine the cAMP concentrations present in heart and lung tissues. Portions of heart and lung were individually weighed and homogenized in 0.1 N HCl (HCl to tissue mass ratio) using a Mini bead beater (Cole Parmer, Vernon Hills, IL, USA). Protein concentrations were analyzed using a Bradford assay, using a Synergy HT plate reader and Gen5 software (BioTek Instruments, Winooski, VT, USA). Heart homogenates were further diluted 1:50 and lung homogenates were further diluted 1:100 using 0.1 N HCl. Using an option provided with the assay, we performed an acetylation step to increase the sensitivity of the ELISA by 10-fold. Then the assay was prepared per the manufacturer's instructions and the experimental samples were plated in duplicate. Optical densities were read at 405 nm using a Synergy HT plate reader and Gen5 software (BioTek Instruments). In assays of heart homogenates, optical densities below 0.3 were taken as 0 pmol/mL. In assays of lung homogenates, optical densities below 0.27 were taken as 0 pmol/mL. Concentration values for cAMP were then normalized to mg of total protein for data reporting.

Adenosinergic Modulation of Vascular Tone in Isolated Aortas

Thoracic aortas were cleaned of surrounding perivascular tissue, cut into 2 mm length rings, and mounted into a DMT 610M multi-channel myograph system (Ann Arbor, MI, USA). Aortic rings were maintained in 37 °C PSS (see above description), bubbled with medical grade breathing air, stretched to a tension yielding 20 mN of force on the transducer, and allowed to relax for 15 min. Then each ring was washed with fresh PSS and re-stretched to a 20 mN force readout. This process was repeated until rings maintained a resting tension at 20 mN of force. Tension was then quickly released to a passive tension yielding 10 mN of force and rings were allowed to equilibrate for 10 min. Aortic rings were assessed for viability via K^+-depolarization using 109 mM K^+PSS (PSS with equal molar substitution of K^+ for Na^+) and basic endothelial function using 1 µM phenylepherine (PE), an α-adrenergic agonist (cat# P6126, Sigma-Aldrich, St. Louis, MO, USA), to contract aortic smooth muscle and 3.0 µM acetylcholine (cat# A6625, Sigma-Aldrich) to stimulate endothelial-dependent relaxation. After an additional 3 × 10-min washout periods, aortic rings were incubated for 20 min with no inhibitor, 10 µM indomethacin—a general COX inhibitor (cat# I7378, Sigma-Aldrich), or 1 mM Nω-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME)—a general NO synthase inhibitor (Cat# N5751, Sigma-Aldrich). Aortic rings were then

stimulated with 1 μM PE for 5 min to allow active stress development to stabilize, followed by cumulative additions of 2-Cl-Adenosine—a stable purinergic adenosine analog (Cat# 3136, Tocirs Bioscience/R&D Systems, Inc., Minneapolis, MN, USA) every 5 min from 0.1 nM to 30.0 μM.

Statistics

Data are expressed as mean ± SEM. Differences were considered significantly different when $p < 0.05$. BAL fluid protein, BAL cell differentials, cAMP concentrations, and maximal aortic responses were analyzed by ANOVA with Tukey's post-test. In Fig. 4, mean data points of concentration–response curves for 2-Cl-adenosine were analyzed by repeated measures ANOVA (marked with $*p < 0.05$ vs. Vehicle). Additionally, a non-linear curve fit was applied to the response profile and analyzed for statistical difference of the resultant fitted lines. This approach allows for the interrogating of complex responses often associated with second messenger systems [27].

Results

BAL Fluid Analysis

The results from fluid protein and cell differential analyses from BAL samples collected 24 h following MWCNT instillation are provided in Table 1. The following results were significantly different than the Vehicle group: (i) mice instilled with C-grade were found to have significantly elevated protein concentration in BALF, increased cellularity, and neutrophilia; (ii) mice instilled with COOH were found to have eosinophilia; (iii) mice instilled with N-doped were found to have significantly elevated cellularity, neutrophilia, and eosinophilia. The extent of eosinophilia found in mice instilled with COOH was significantly different from that of the C-grade group. The extent of neutrophilia found in mice instilled with N-doped was also significantly different from the COOH group.

Lung Histology

Representative histological images of the left lung, 24 h after MWCNT instillation are shown in Fig. 1. Histological analysis had consistent results with BAL analysis. When taking histological lung samples from the Vehicle group (Fig. 1a) as a baseline comparison, lung samples from the C-grade group showed some increased cellularity and wall thickening (Fig. 1b). Histological lung samples from the COOH group appeared to fall between the Vehicle and C-grade groups in terms of cellularity and wall thickening (Fig. 1c). When compared to Vehicle, C-grade, and COOH, the N-doped group appeared to have the highest degree of pulmonary cellularity and wall thickening in response to MWCNT instillation (Fig. 1d).

Cardiac I/R Injury

We verified that the CD-1 ICR mouse model would respond to MWCNT instillation with expansion of cardiac I/R injury, as we have previously reported for C57BL/6 mice [5]. The sizes of myocardial infarctions generated by cardiac I/R, 24 h following MWCNT exposure are presented in Fig. 2 as a percent of the zone at risk for infarction. Myocardial infarct size was significantly larger following I/R in the C-grade, COOH, and N-doped groups when compared to the Vehicle group by 33%, 60%, and 87%, respectively. Myocardial infarct size in the COOH and N-doped groups was also significantly larger than the infarction in the C-grade group by 21% and 41%, respectively. Myocardial infarct size in the N-doped group was also significantly larger by 16% than the infarct size in the COOH group.

Heart and Lung cAMP Concentrations

Data from cAMP measurements in heart and right lung tissues collected 24 h after MWCNT instillation are reported in Fig. 3. Analysis of heart tissue revealed that cAMP concentrations were significantly decreased in the C-grade and N-doped groups (17.4% and 13.7%, respectively) when compared to the Vehicle group (Fig. 3a). Heart cAMP

Table 1 Bronchoalveolar lavage fluid analysis

Bronchoalveolar lavage fluid analysis						
	[Protein] μg/mL	Total cells $\times 10^4$	Macrophages $\times 10^4$	Neutrophils $\times 10^3$	Eosinophils $\times 10^3$	Lymphocytes $\times 10^3$
Vehicle	167 ± 27	15.9 ± 1.1	14.4 ± 1.2	8.1 ± 4.1	1.1 ± 0.4	2.7 ± 0.6
C-grade	409 ± 40*	39.7 ± 3.7*	21.4 ± 2.0	148 ± 25*	11.2 ± 1.1	4.3 ± 1.2
COOH	255 ± 40	26.5 ± 2.3	14.5 ± 1.2	41.4 ± 2.0	69.1 ± 9.0*,†	5.2 ± 1.0
N-doped	324 ± 60	36.2 ± 6.4*	13.7 ± 2.1†	163 ± 50*,‡	42.2 ± 17*	6.1 ± 1.0
	Mean ± SEM ($n = 6$)					

$*p < 0.05$ vs. Vehicle; $^{\dagger}p < 0.05$ vs. C-grade; $^{\ddagger}p < 0.05$ vs. COOH

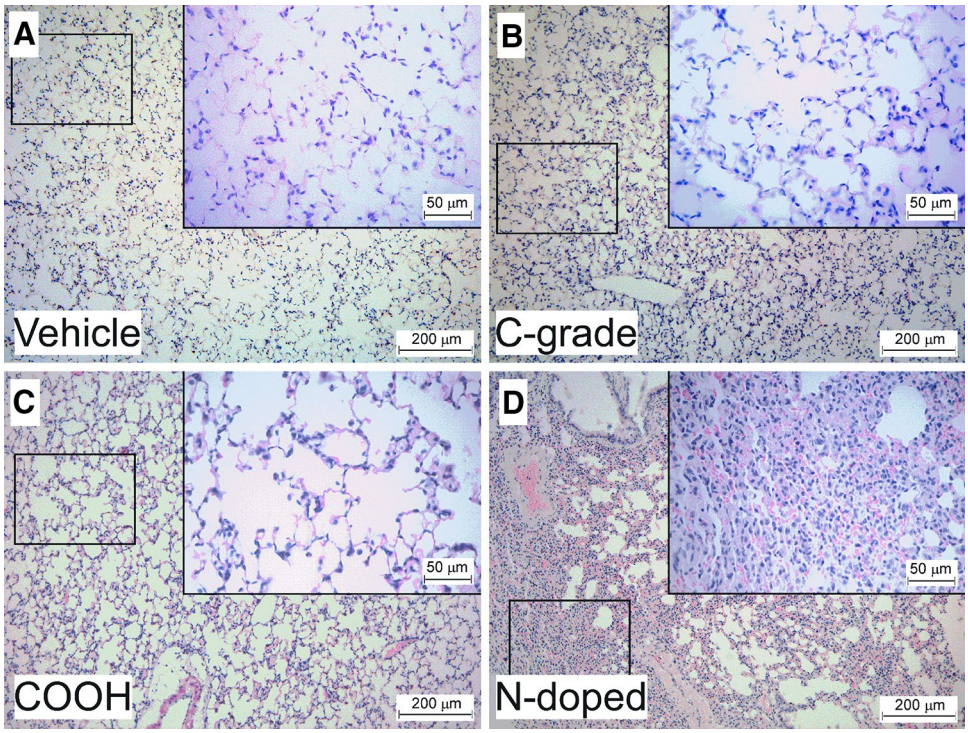

Fig. 1 Lung histology. Left lungs were collected 24 h following Vehicle or MWCNT instillation and examined histologically by H&E staining in order to visually assess pulmonary responses to MWCNT. Images were collected using the 10× objective and inlays were collected using the 40× objective. **a** Representative images of a left lung tissue collected from mice instilled with Vehicle. **b** Representative images of lung tissue collected from mice instilled with C-grade. **c** Representative images of left lung tissue collected from mice instilled with COOH. **d** Representative images of left lung tissue collected from mice instilled with N-doped

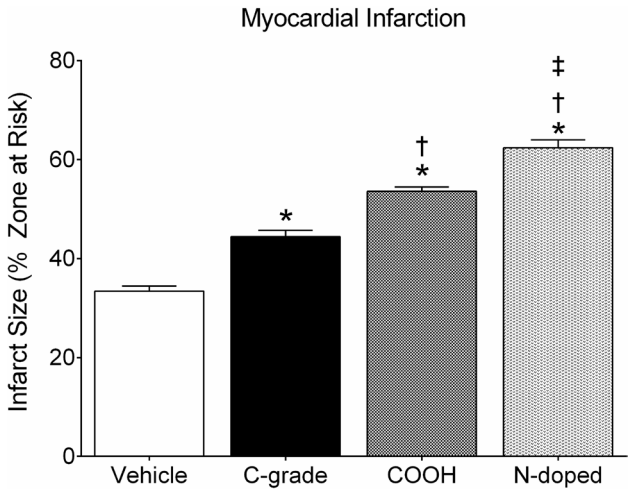

Fig. 2 Cardiac I/R injury. We tested the hypothesis that 24 h after MWCNT instillation cardiac I/R injury would result in expansion of myocardial infarction. Myocardial infarct sizes from CD-1 mice instilled with Vehicle, 100 µg C-grade, COOH, or N-doped are presented as a percent of the zone at risk. $^{*}p < 0.05$ vs. Vehicle; $^{\dagger}p < 0.05$ vs. C-grade; $^{\ddagger}p < 0.05$ vs. COOH as determined by ANOVA. $N = 5$ for all groups

levels were not different between the COOH and Vehicle groups. We also examined lung tissue for changes in cAMP following MWCNT instillation but found no significant differences between groups (Fig. 3b).

Adenosinergic Modulation of Aortic Tone

Aortic adenosinergic modulation of α-adrenergic (phenylephrine—PE) force generation is presented in Fig. 4. We conducted these experiments either in the presence or absence of the general COX inhibitor indomethacin (10 µM) or the NO synthase inhibitor L-NAME (1 mM). Use of a best-fit comparison of non-linear fits of the cumulative 2-Cl-adenosine response curves revealed significant differences between the MWCNT groups and the Vehicle group when no inhibitors were present (Fig. 4a). The same analysis of best fit comparison of the cumulative response curves revealed a significant difference between the N-doped and C-grade groups with no inhibition. When aortic 2-Cl-adenosine best fit responses lines were assessed in the presence of 10 µM indomethacin no significant differences were detected between any groups (Fig. 4b). When aortic 2-Cl-adenosine best fit responses lines were compared in the presence of 1 mM L-NAME, the fits of the COOH and C-grade groups were significantly different from the Vehicle group (Fig. 4c). 2-Cl-adenosine responses in the COOH group were also significantly different from the Vehicle group when compared by repeated measures ANOVA. Aortic 2-Cl-adenosine responses of the N-doped group were not significantly different from Vehicle group.

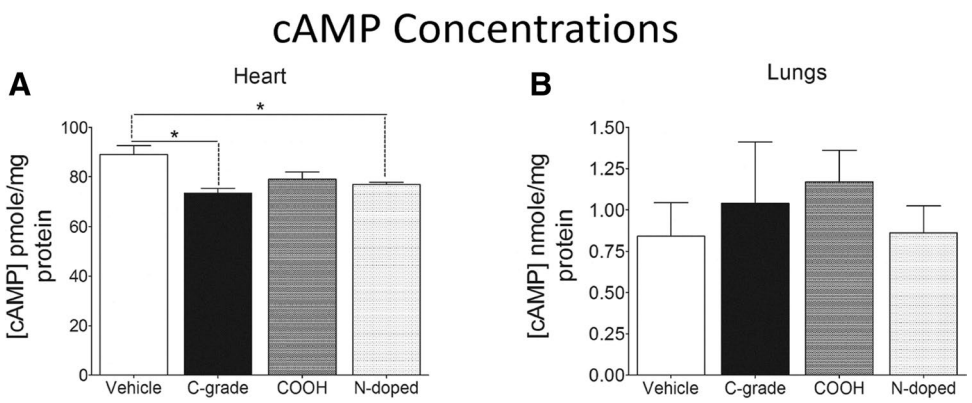

Fig. 3 Heart and lung cAMP concentrations. Heart and right lung tissues were collected from mice 24 h after instillation with Vehicle or MWCNT and assessed for cAMP concentrations via ELISA. **a** cAMP concentrations in heart tissue collected from mice instilled with Vehicle ($n=6$), C-grade ($n=5$), COOH ($n=5$), or N-doped ($n=6$) normalized to total protein. **b** cAMP concentrations in lung tissue collected from mice instilled with Vehicle ($n=8$), 100 μg C-grade ($n=4$), COOH ($n=4$), and N-doped ($n=8$) normalized to total protein. *$p<0.05$ vs. Vehicle

Peak Aortic Responses

The peak aortic constrictor responses to PE and relaxation response to 2-Cl-adenosine are reported in Fig. 5. Despite some separation in the mean constrictor stress responses generated by PE stimulation, there were no significant differences between MWCNT treatment groups within any of the inhibitor groups (Fig. 5a). We did find eNOS inhibition with L-NAME enhanced the PE stress responses in the Vehicle and COOH groups when compared to PE stress when no inhibitor was present. Maximum aortic relaxation achieved with 30 μM 2-Cl-adenosine stimulation was not different between groups when no inhibitors were present or in the presence of the COX inhibitor indomethacin (Fig. 5b). However, maximum 2-Cl-adenosine relaxation was blunted by 80% in the N-doped group compared to the Vehicle group when aortas were incubated with 1 mM L-NAME a NOS inhibitor.

Discussion

The key finding in this study is that pulmonary exposure to modified MWCNT influences cardiovascular adenosinergic responses in the CD-1 ICR mouse model promoting constriction in the face of adrenergic stimulation. In particular, intratracheal instillation of modified MWCNT diminishes cAMP in myocardial tissue and adapts adenosine modulation of aortic smooth muscle tone via imbalanced NO and COX mechanisms. We found that these endpoints were uniquely impacted depending on exposure to either C-grade, COOH, and N-doped MWCNT. Moreover, responses in the lung, as well as cardiac I/R injury are also dependent on the type of MWCNT encountered during exposure. The CD-1 mouse strain has been used for receptor subtype knockout studies of adenosine receptor function [11, 28] and allows the comparison of cardiac I/R injury after MWCNT instillation between this study and those we previously reported in the C57BL/6 model [5].

Accumulating evidence as recently reviewed by Abukabda et al. [29] and Stone et al. [30] suggests that engineered nanomaterials detrimentally impacts cardiovascular system health by a diversity of postulated modes of action. Many of the modes are borne out of the extensive work reported on ambient particulate matter exposures [31] and include central nervous system effects, pulmonary and systemic inflammation responses, and possible direct particle effects. A common target for the all of these modes include the endothelium. This is a tissue critical in the regulation of vascular function to all organs by autocrine, paracrine, and hormonal signaling cascades. The endothelium may be sensitive to the physiochemical distinction of the MWCNT impacting a NO/reactive oxygen species imbalance and creating a tissue environment that promotes injury through thrombosis, vascular dysfunction, and autonomic dysreflexia (reviewed in [29–31]).

Exposure to MWCNT produces unique airway responses and I/R-induced myocardial infarct size depending on the modification, which is consistent with our previous report [5]. In both these studies, infarct sizes graded in size with smallest to largest in the order of Vehicle < C-grade < COOH, with N-doped. In the present study, mice instilled with C-grade had significant increases in BALF protein, cellularity, and neutrophilia compared to the Vehicle groups. Both studies showed that N-doped exposure increased cellularity and neutrophilia compared to the Vehicle instillation. This type of cellularity/neutrophilia has also been demonstrated with MWCNT exposure in mice [32] and

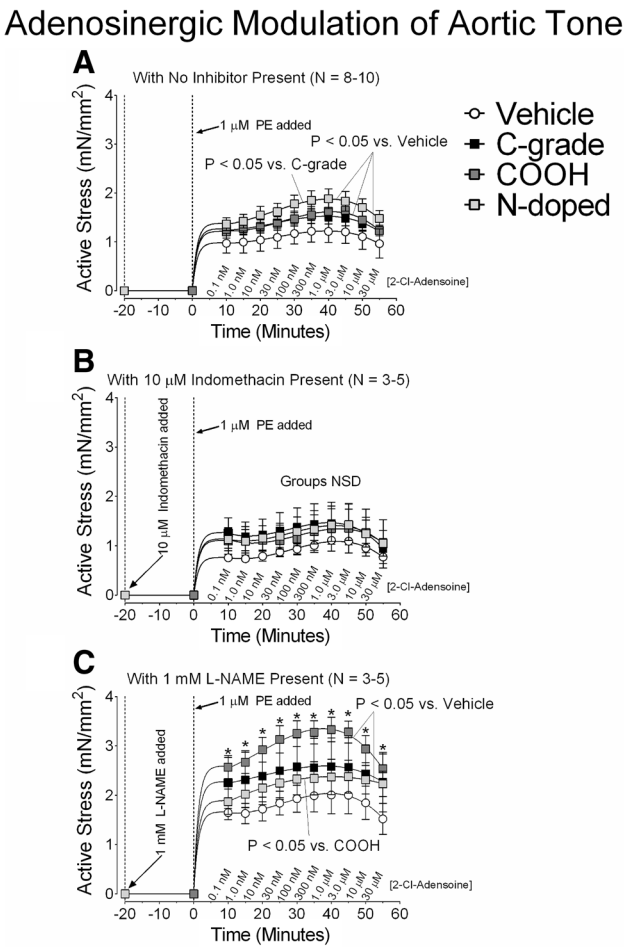

Fig. 4 Adenosinergic modulation of aortic tone. Isolated rings of thoracic aorta were stimulated with 1 μM phenylepherine (PE) for 5 min followed by additions of 2-Cl-adenosine every 5 min to generate cumulative concentration–responses from 0.1 nM to 30 μM. **a** Concentration–response profiles with NO Inhibitor present ($n=8$–10). **b** Concentration–response profiles with 10 μM general cyclooxygenase (COX) inhibitor indomethacin present ($n=3$–5). **c** Concentration–response profiles with 1 mM nitric oxide synthase (NOS) inhibitor L-NAME present ($n=3$–5). $p<0.05$ for comparisons of the nonlinear fits of the treatment response curve was determined by best fit regression analysis [24]. *$p<0.05$ vs. Vehicle for mean data reported as done by repeated measures ANOVA with Bonferroni post-test. ($n=3$–10)

rats [33]. One contrast between our two studies were that CD-1 mice did not present with increased BALF protein concentration after N-doped exposure, as was seen with the C57BL/6 mice [5]. We did find eosinophilia in the COOH and N-doped groups, which we have reported in rats 24 h after instillation of C-grade MWCNT [34] and 60-carbon fullerenes [35].

One of the key findings here is that exposure to modified MWCNT decreases cardiac cAMP concentrations. More specifically, we found that cardiac cAMP was significantly lower in the C-grade and N-doped groups when compared to the Vehicle group, with the mean value of cAMP in the COOH group also being lower (but not statistically different) than that of the Vehicle group. Decreased tissue cAMP concentration can result from excess hydrolysis of cAMP by phosphodiesterases, decreased synthesis by adenylate cyclase, or by extrusion from cardiomyocytes by ATP-binding cassette transporters [36]. One important function of cAMP is activation of protein kinase A, which has reported involvement in I/R cardioprotection [37, 38]. The myocyte cyclic nucleotide system is also important for appropriate heart responses to functional regulation by the autonomic nervous system, endocrine system, and autocrine/paracrine systems via G-protein coupled receptor activation [39]. Thus, reductions in cAMP concentrations after MWCNT exposure may contribute to cardiac injuries and blunting of cAMP-dependent heart function.

We also found adaptations in aortic responsiveness to adenosine following exposure to all three types of MWCNT but the responses were unique depending on the surface modifications. Best-fit analysis of the fitted curves revealed that that N-doped and COOH exposure augmented cumulative response profile to 2-Cl-adenosine. These alterations were prevented by treating aortic rings with indomethacin, a general COX inhibitor. However, NOS inhibition produced enhanced aortic contractile and relaxation responses to 2-Cl-adenosine in the COOH group but blunted the relaxation responses in the N-doped group. Extracellular adenosine is an important arm of the cardiovascular adenosinergic system, which includes the regulation of heart rate, myocardial contractility, coronary flow, and vascular tone [6], so one may suggest alterations in adenosinergic signaling after MWCNT exposure may impact functions beyond the vascular system. Similar alterations in vascular NO and COX signaling following MWCNT instillation have been reported, including blunted endothelium-dependent dilation [40–42] and enhanced vasocontraction via COX [28]. While other mouse models have reported an augmented aortic constriction to adenosine in the face of modulated NO/COX signaling of the endothelium [23–25]. Given that myocardial ischemia can cause the release of adenosine [22, 43], altered vascular responses to extracellular adenosine may have played a role in the expansion of cardiac I/R injury after exposure to the COOH and N-doped forms of MWCNT. When considered together, these findings indicate that MWCNT exposure can alter adenosinergic signaling and that nitrogen-doping and carboxylation modifies these responses, which may make predicting health outcomes of exposure more difficult.

Before drawing final conclusions from our findings, some limitations must be acknowledged. First, we have used a high dose of MWCNT for instillation in this study. We have previously reported dose-response and time-course findings associated with the MWCNT used

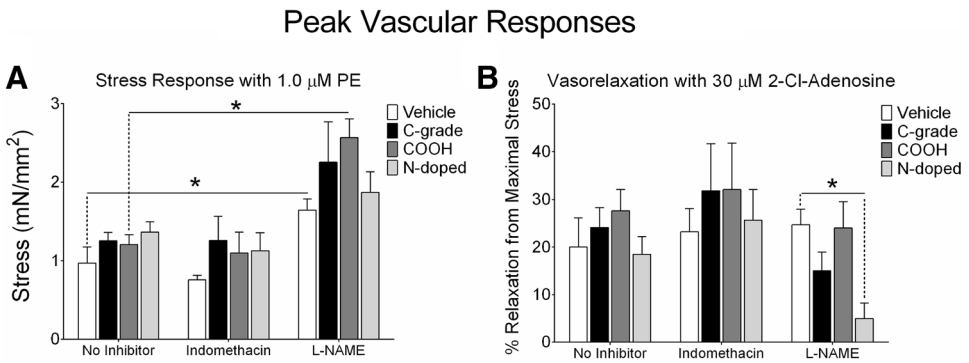

Fig. 5 Aortic contraction with phenylephrine (PE) and relaxation with 2-Cl-adenosine. **a** Maximal aortic stress generated 5 min following stimulation with 1 μM PE, just prior to the start of 2-Cl-adenosine protocols ($n = 3–10$). **b** The maximal aortic vasorelaxation generated by stimulation with 30 μM 2-Cl-adenosine taken as a function of peak stress generated during experimental protocols with PE + 2-Cl-adenosine ($n = 3–10$). *$p < 0.05$ by Two-way ANOVA with Bonferroni post-test

in this study [5]. The purpose of our present study was to investigate the impact of MWCNT exposure on adenosinergic cardiovascular endpoints and further determine if the responses were unique based on form of modified MWCNT. We selected the high dose of MWCNT used in our previous study to interrogate a mechanism of dysfunction, future efforts need to be undertaken to determine any dose- and time-dependent aspects of altered adenosinergic signaling contributing to the apparent cardiovascular detriment. We have also examined vascular responses in aortic tissue as general assessments of arterial endothelial and smooth muscle function as this tissue is routinely used to support studies examining function in other arteries. However, it will be informative to examine adenosine responsiveness in small resistive arteries. This question will be particularly relevant in assessing the role of MWCNT exposure on the adenosinergic signaling in coronary vasculature due to is prominent role in protecting the myocardium form ischemic damage.

In conclusion, modified versions of MWCNT can uniquely alter cAMP concentrations in the heart and influence aortic adenosinergic responsiveness via dependence on NO and COX signaling. The impact of MWCNT exposure on cAMP concentrations in the heart could possibly disrupt other signaling mechanisms that rely on cyclic nucleotides. The alterations in vascular adenosinergic signaling following exposure to surface-modified MWCNT could cause circulatory supply and demand mismatching. The combination of impacted adenosinergic systems after exposure to C-grade and modified MWCNT could narrow cardiovascular homeostasis to a degree that episodes like cardiac I/R may contribute to the observed exacerbated injury. Given that the utility of MWCNT is expanding across industries, a more thorough understanding of physiological consequences occurring because of exposure is relevant.

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Compliance with Ethical Standards

Conflict of interest The authors report no conflicts of interests related to the research reported in this manuscript.

Research Involving Animals Rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were approved and in accordance with the ethical standards of the Institutional Animal Care and Use Committee at East Carolina University (Greenville, NC, USA).

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