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The Effects of Subacute Inhaled Multi-Walled Carbon Nanotube Exposure on Signaling Pathways Associated with Cholesterol Transport and Inflammatory Markers in the Vasculature of Wild-Type Mice.

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Abstract

Exposure to multi-walled carbon nanotubes (MWCNTs) has been associated with detrimental cardiovascular outcomes; however, underlying mechanisms have not yet been fully elucidated. Thus, we investigated alterations in proatherogenic and proinflammatory signaling pathways in C57Bl6/ mice exposed to MWCNTs (1 mg/m³) or filtered air (FA-Controls), via inhalation, for 6 hr/day, 14d. Expression of mediators of cholesterol transport, namely the lectin-like oxidized low-density lipoprotein receptor (LOX)-1 and ATP-binding cassette transporter (ABCA)-1, inflammatory markers tumor necrosis factor (TNF)- α and interleukin (IL)-1 β /IL-6, nuclear-factor kappa-light-chain-enhancer of activated B cells (NF- κ B), intracellular/vascular adhesion molecule(s) (VCAM-1, ICAM-1), and miRNAs (miR-221/-21/-1), associated with cardiovascular disease (CVD), were analyzed in cardiac tissue and coronary vasculature. Cardiac fibrotic

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Availability of Data and Material.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions.

GD helped design the backend experiments and conducted the experimental procedures, analysis, and writing of the manuscript. JL assisted with the tissue collection and preparation of tissues for all histological and molecular endpoints, as well assisted with revisions of the manuscript. CF completed the pulmonary experiments and data analysis. JDM designed and managed inhalation MWCNT exposures. AKL designed the backend experiments, assisted with analysis and interpretation of data, and critically revised the manuscript. All authors read and approved the manuscript.

Conflict of Interest.

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deposition, matrix-metalloproteinases (MMP)-2/9, and reactive oxygen species (ROS) were also assessed. MWCNT-exposure resulted in increased coronary ROS production with concurrent increases in expression of LOX-1, VCAM-1, TNF- α , and MMP-2/9 activity; while ABCA-1 expression was downregulated, compared to FA-Controls. Additionally, trends in fibrotic deposition and induction of cardiac TNF- α , MMP-9, I κ B Kinase (IKK)- α/β , and miR-221 mRNA expression were observed. Analysis using inhibitors for nitric oxide synthase or NADPH oxidase resulted in attenuated coronary ROS production. These findings suggest that subacute inhalation MWCNT-exposure alters expression of cholesterol transporter/receptors, and induces signaling pathways associated with inflammation, oxidative stress, and CVD in wild-type mice.

Graphical Abstract

Keywords

MWCNT; coronary arteries; miRNA; ROS; LOX-1

1. Introduction

Cardiovascular disease (CVD), such as coronary artery disease (CAD), is the leading cause of death for people of most ethnicities in the United States and worldwide (NHLBI, NIH). While many of the contributing factors involved in the etiology of CVD, such as genetics and diet, have been extensively characterized, much less is understood on whether inhalation exposure to nanomaterials may also contribute to CVD. Multi-walled carbon nanotubes (MWCNTs), which are classically defined as multiple concentric cylinders of carbon rings, are generated from numerous environmental sources such as traffic emissions, atmospheric nucleation processes, and semi-volatile materials, as well as mass-produced for industrial and medicinal uses (Lam et al., 2006). Importantly, aerosolized MWCNTs are of potential concern as they may enter the environment as suspended particulate matter (PM) that can be inhaled (Lam et al., 2006). Previous inhalation toxicity studies report that some MWCNTs, at relatively high doses, can induce an inflammatory response in the lungs, resulting in activation of leukocytes (Manke et al., 2013; Mitchell et al., 2009). However, the role of MWCNT-exposure in the etiology of vascular disease remains less clear, as conflicting results have been reported in the literature (Cao et al., 2014; Han et al., 2015). Thus, further characterization of inhaled MWCNT exposure-mediated outcomes on the vasculature, in

addition to the identification of potential signaling pathways involved, is necessary to fully understand the potential role of MWCNT-exposure in vascular disease.

Atherosclerotic plaque-induced occlusion of the coronary arteries may lead to angina, heart attack, restenosis, vascular fibrosis, and vascular smooth muscle cell (VSMC) hypertrophy; all of which are typically associated with endothelial dysfunction and/or alterations in cholesterol transport (Pirillo et al., 2013). Low-density lipoprotein (LDL) is a carrier of cholesterol that increases proportionally relative to the amount of endogenous cholesterol synthesized and dietary cholesterol absorbed (Pirillo et al., 2013). In the presence of reactive oxygen species (ROS), circulating LDL is often oxidized forming a reactive oxidized LDL (ox-LDL) molecule that will bind and internalize in vascular endothelial cells (ECs) via lectin-like oxidized low-density lipoprotein receptor (LOX)-1, which is the primary ox-LDL receptor present on ECs (Pirillo et al., 2013). When internalized into the vascular endothelium, ox-LDL can result in initiation and/or progression of atherosclerotic plaque growth (Pirillo et al., 2013).

Alternatively, ATP Binding Cassette Transporter (ABCA)-1 is a transporter protein that plays a protective role in the vascular endothelium via reverse cholesterol transport of LDL (rev. in Yin et al., 2010). However, proinflammatory cytokines such as tumor necrosis factor (TNF)- α and Interleukin (IL)-1 β , can attenuate protein levels and promoter activity of ABCA-1 via ROS- and nuclear factor (NF)- κ B- dependent pathways (rev. in Yin et al., 2010).

Inflammatory and vasoactive factors are also associated with vascular injury and progression of CVD. For example, endothelin (ET)-1 is a vasoconstrictive and mitogenic peptide, which is often found upregulated in CVD, including atherosclerosis (Li et al., 2013). Increased ET-1 expression often exacerbates vascular disease-states, as its expression is associated with mediating production of ROS, increased expression of intercellular and vascular adhesion molecule(s)-1 (ICAM-1 and VCAM-1, respectively), and also induction of matrix metalloproteinase (MMP)-2/9 activity in the vasculature, which can lead to the disruption of the extracellular matrix (ECM) and eventually contribute to atherosclerotic plaque growth and/or rupture (Liu et al., 2006; Li et al., 2013). Proatherogenic cytokines capable of inflammation such as interleukin (IL)-6, IL-1 β , and TNF- α have also been identified as key mediators in atherogenesis (Tousoulis et al., 2016). Furthermore, signaling factors within the TNF superfamily, namely TNF- α and osteoprotegerin (OPG), have been previously identified to be involved in canonical and alternative activation of NF- κ B pathways, respectively (Lawrence, 2009). The canonical NF- κ B pathway involves initial activation from TNF- α , leading to I κ B kinase (IKK)- β phosphorylating negative inhibitor (I) κ B- α for degradation. In turn, this activates the p65/RelA complex; which will lead to the regulation of proinflammatory and cell survival gene expression (Lawrence, 2009). Alternatively, stimuli such as receptor activator of nuclear factor kappa-B ligand (RANKL), namely OPG, acts as a stimulus to IKK- α ; this allows for the independent phosphorylation of p100 to activate the p52/RelB complex, which eventually leads to changes gene expression in respect to adaptive immunity (Lawrence, 2009). NF- κ B, and the signaling pathways it is involved in, plays a critical role in nearly all stages of plaque growth in atherosclerosis and is often considered as a target for therapeutic intervention (Tousoulis et al., 2016).

More recently, alteration in expression of certain microRNAs (miRNAs) has been associated with vascular disease. For example, increased miRNA (miR)-221 expression is associated with changes in vascular smooth muscle cell (VMSC) and vascular EC proliferation, production of proinflammatory cytokines such as TNF- α and IL-6, and altered nitric oxide (NO) production (Liu et al., 2010; Zhao et al., 2016). Additionally, miRNA (miR)-21 expression is significantly higher in atherosclerotic plaques vs. that observed in healthy arteries, and is associated with increased NO production and nitric oxide synthase (NOS) phosphorylation (Weber et al., 2010). Finally, downregulation of miRNA (miR)-1 has been linked to cardiac hypertrophy (Feinberg and Moore, 2016).

Characterization of initial inflammation and signaling cascades that follow from an inhalation exposure to MWCNTs has not been thoroughly explored. As such, we investigated the hypothesis that subacute inhalational exposure to MWCNT alters signaling pathways associated with inflammation and atherogenesis, using a healthy wildtype mouse model. Importantly, these inhalational exposures are the same dose (1 mg/m³) and duration (14d) as those previously reported that showed no meaningful results in lung inflammation or pathology, but did report activated inflammatory signaling in the spleen (Mitchell et al., 2007) and suppressed systemic immune function (Mitchell et al., 2009). While higher than that expected from an environmental or occupational inhalation exposure utilizing the same dose, route of exposure, and animal model allows us to further investigate the systemic effects of inhalation MWCNT-exposures.

2. Methods

2.1 MWCNT inhalational exposure in C57Bl/6 mice

As previously described, the MWCNTs used for this study have been extensively characterized for structure, purity, and deposition (Mitchell et al., 2007, 2009). Male C57Bl/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) at approximately 8 weeks of age and quarantined for 2 weeks prior to exposure initiation. Mice were exposed for 6 h/day for 14 consecutive days to atmospheres containing 1 mg/m³ MWCNTs or control air (n=7 per group). The total amount of MWCNTs deposited in the lung is estimated to be 0.5 mg/kg at 1 mg/m³ (approximately 12.5 μ g total). Estimation of the dose was calculated as previously described in Mitchell et al., 2007. Importantly, the dose and duration for these MWCNT-exposures were based on previous studies that reported altered systemic inflammatory signaling an immune function in the absence of overt pulmonary toxicity (Mitchell et al., 2007, 2009). Mice were housed in standard shoebox cages within an Association for Assessment and Accreditation of Laboratory Animal Care International-approved rodent housing facility (2 m³ exposure chambers) for the entirety of the study, which maintained a constant temperature (20–24°C) and humidity (30–60% relative humidity). Mice had access to chow and water *ad libitum* throughout the study period, except during daily exposures when chow was removed. All procedures were approved by the Lovelace Biomedical and Environmental Research Institute's Animal Care and Use Committee (AAALAC-accredited; USDA-registered facility) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2 Tissue collection

Upon completion of the 14d exposure, animals were sacrificed within 14–16 hrs after their last exposure, and tissues were collected. To minimize any suffering, mice were anesthetized with Euthasol (390 mg pentobarbital sodium, 50 mg/ml phenytoin sodium; diluted 1:10 and administered at a dose 0.1 ml per 30 g mouse) and euthanized by exsanguination. The heart was treated using HistoChoice tissue fixative (97060–930, VWR, Radnor, Pennsylvania) with an additional 30% Sucrose-PBS solution. Fixed hearts were dissected and split, where the superior 2/3 of the heart was embedded in OCT (VWR Scientific, West Chester, PA) and frozen on dry ice and the remaining 1/3 of the heart (ventricles) was immediately snap frozen in liquid nitrogen for molecular assays. All tissues were stored at -80°C until processed for analysis.

2.3 Real time RT-PCR analysis

Total RNA was isolated from 30 mg of left ventricular heart tissue using an AllPrep DNA/RNA/miRNA isolation kit (Qiagen, Germantown, MD), following the manufacturer protocol. Isolated RNA was then synthesized into cDNA using iScript Reverse Transcription Supermix for reverse transcription (Bio-Rad, Hercules, CA). Synthesized cDNA was used for real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to determine transcriptional expression of messenger RNA (mRNA) using forward and reverse primers for LOX-1, ABCA-1, ICAM-1, VCAM-1, TNF- α , IL-6, IL-1 β , ET-1, MMP-9, OPG, TGF- β 1, RelA, RelB, IKK- α/β , and I κ B- α (Supplementary Table 1) using Bio-Rad SSo SYBR green detection (Bio-Rad), following the manufacturer's protocol. Isolated RNA, in conjunction with the miScript II RT kit (Qiagen), produced cDNA specific to miRNA detection, following manufacturer's instruction. Primers for detecting the transcriptional expression of miR-1, miR-21, and miR-221 using miScript SYBR Green PCR kit (Qiagen) with specific primers for each miRNA (Supplementary Table 1). Results for both mRNA and miRNA RT-qPCR were calculated/normalized, as previously described by our laboratory (Lund et al., 2009, 2011). An n=7 per group were used for real time PCR analysis.

2.4 Double immunofluorescence staining

The ventricles from the study mice were embedded in OCT, frozen, and sectioned on a cryostat at 7 μm thickness. Slides were then prepared for double immunofluorescence microscopy, as previously described by our laboratory (Lund et al., 2011). Primary antibodies used: LOX-1 (OLR-1 Receptor) (ab81709, Abcam, Cambridge, Massachusetts), ABCA-1 [cholesterol efflux regulatory protein (CERP)] (ab18180, Abcam), VCAM-1 (ab1340478, Abcam), TNF- α (ab6671, Abcam), NF- κ B (ab86229, Abcam), von Willebrand factor [(vWF); endothelial cell-specific marker] (ab11713, Abcam). Secondary antibodies used: Alexa Fluor 488 donkey anti-sheep IgG (H+L) Cross-Adsorbed Secondary Antibody (#A-11015, ThermoFisher Scientific, Richardson, Texas) and Alexa Fluor 555 Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody (#A-21422, ThermoFisher Scientific). Nucleic staining used: Hoescht 33342 (#89166–026, VWR) and incubated for 1 min before rinsing. Slides were then cover-slipped and imaged by fluorescent microscopy at 40 $\times$, using the appropriate excitation/emission filters, digitally recorded, and analyzed by image densitometry to measure vessel fluorescence of primary antibody (only) and

subtracting background within the lumen of vessels using ImageJ (NIH, Bethesda, MD). A minimum of 3–5 coronary vessels on each section (2 sections per slide), 3 slides and $n=3$ per group were processed/analyzed taking individual fluorescent images of red (protein of interest, primary antibody), green (endothelial expression, VWF), and blue (Nuclei expression, Hoescht) and an overlay image combining all source images.

2.5 Dihydroethidium (DHE) staining

Sections of hearts (embedded in O.C.T. and cryosectioned at 7 μm) were immediately processed through DHE staining, as previously described by our laboratory (Lund et al., 2011). Select antagonists that inhibit ROS pathways were used to determine the source(s) of nanomaterial-mediated ROS induction. Antagonists were applied to the slides and incubated at 37°C for 30 minutes prior to DHE staining. Inhibiting reagents include Apocynin (5 $\mu\text{mol/L}$) for NADPH oxidase (NOX) inhibition, Sodium diethyldithiocarbamate trihydrate [(S-DECT)(10 $\mu\text{mol/L}$)] for Superoxide Dismutase (SOD) inhibition, BAY 11–7085 (1 $\mu\text{mol/L}$) for NF- κB inhibition, and Allopurinol (10 $\mu\text{mol/L}$) for xanthine oxidase (XO) inhibition, and L-N^G-Nitroarginine [(L-NNA) (10 $\mu\text{mol/L}$)] for nitric oxide synthase (NOS) inhibition. Resulting ethidium staining was visualized by fluorescent microscopy at 40 $\times$, digitally recorded, with individual fluorescent images of red (intensity indicating levels of ROS), and analyzed by image densitometry using Image J software (NIH). A minimum of 3–5 coronary vessels on each section (2 sections per slide), 3 slides and $n=3$ per group were processed/analyzed.

2.6 *In situ* zymography

MMP-2/9 activity was analyzed on frozen serial heart sections (7 μm thick), with coronary arteries present, as previously described by our laboratory (Lund et al., 2011; Oppenheim et al., 2013). Gelatinase/collagenase activity was analyzed using fluorescent microscopy and densitometry calculated using white/black images and quantified using Image J software (NIH). The analysis was performed on an $n=3$, 3 regions per section, 3–5 samples per group, with background fluorescence (outside of the coronary vessel) subtracted from each section prior to comparison.

2.7 Masson's trichrome staining

Histology slides were prepared using Masson's trichrome staining to determine the amount of collagen present within the heart using Trichrome stain kit (HT15-K1, Sigma Aldrich Sigma Aldrich, St. Louis, Missouri), following the manufacturer's protocol. Collagen fibers stain blue, nuclei stain black, and ventricular muscle tissue stains red. Treated slides were cover-slipped and evaluated using an EVOS Fl microscope. A minimum of 3–5 coronary vessels on each section (2 sections per slide), 3 slides, and $n=3$ per group were analyzed using color deconvolution to isolate collagen staining. Image intensity and total collagen per unit area were calculated using CellProfiler (Lamprecht et al., 2007).

2.8 Statistics

Data are expressed as mean $\pm$ SEM. A Student's t-test was used for statistical analysis between exposed and control samples involving only direct comparisons between control

and MWCNT exposed animals. A two-way ANOVA was used to compare exposed and control samples $\pm$ inhibitors for the dihydroethidium staining protocol, a $\dagger p < 0.050$ was considered statistically significant reductions in MWCNT-induced DHE+inhibitor group, compared to the non-inhibitor treated MWCNT exposure group (baseline ROS). Statistical analyses were conducted using Sigma Plot 12.5 (Systat, San Jose, CA). A $p < 0.050$ was considered statistically significant for all endpoints analyzed.

3. Results

3.1 Characterization of collagen fibrotic deposition of MWCNT-exposed mice

We utilized Masson's Trichrome staining to determine whether inhalation exposure to MWCNTs resulted in an increase in ventricular fibrotic deposition. Compared to filtered air control (FA-CT) animals (Fig. 1A), perivascular fibrotic intensity showed a statistically significant increase in MWCNT-exposed animals (Fig. 1B, $p < 0.050$), but analysis of total collagen per unit area was not statistically significant; quantitative comparison between groups is represented in Fig. 1C and 1D, respectively.

3.2 Characterization of matrix-metalloproteinase expression from MWCNT exposure

As MMP-2/9 (gelatinase) activity is commonly found upregulated in vascular disease; we examined MMP activity ($-2/9$) and expression (-9) upon exposure to MWCNTs, in the coronary vasculature and heart homogenate using *in situ* zymography and RT-qPCR, respectively. *In situ* zymography revealed that, compared to FA-CT animals (Fig. 2A), coronary arteries from MWCNT-exposed C57Bl/6 mice showed a statistically significant increase in MMP-2/9 activity (Fig. 2B, $p < 0.050$); quantitative comparison between groups is represented in Fig. 2C. Furthermore, transcriptional expression of cardiac MMP-9 was found to be increased in MWCNT-exposed animals (Fig. 2D, $p < 0.050$).

3.3 Detection of common inflammatory and atherogenic factors in MWCNT exposed mice

To determine whether inhalation exposure to 1 mg/m^3 of MWCNT over 14d caused alterations in vascular adhesion and inflammatory signaling pathways in the heart, we investigated the expression of factors associated with vascular disease, namely VCAM-1, ICAM-1, ET-1, and TGF- β 1; in addition to proinflammatory cytokines TNF- α , IL-6, and IL-1 β , via RT-qPCR. Compared to FA-CT mice, we observed no change in expression of cardiac ICAM-1 mRNA (Fig. 3A), and only a modest increase in VCAM-1 mRNA and ET-1 mRNA transcript (Figs. 3B and 3C, respectively), albeit not statistical, in MWCNT-exposed C57Bl/6 mice. Furthermore, TGF- β 1 did not show statistically significant alterations in expression in MWCNT-exposed animals (Fig. 3D). When analyzing inflammatory factors, MWCNT-exposure resulted in a significant increase in cardiac TNF- α mRNA expression, compared to FA-CT mice (Fig. 3E, $p < 0.050$); whereas expression of cardiac IL-6 mRNA (Fig. 3F) and IL-1 β mRNA (Fig. 3G) were not statistically different across study groups. We also used double immunofluorescence microscopy to confirm levels of VCAM-1 and TNF- α within the coronary vasculature, since they were either heightened or statistically significant from RT-qPCR analysis, respectively. VCAM-1 protein expression was found to be increased in coronary vessels when comparing FA-CT animals (Figs. 4A-C) to MWCNT-exposed animals (Figs. 4D-F, $p < 0.050$). Quantitative comparison between groups shown in

Fig. 4G. Furthermore, coronary vessels stained for TNF- α were also significantly higher, compared to FA-CT animals (Figs. 5A-C), in MWCNT-exposed mice (Figs. 5D-F, $p < 0.050$). Graphical comparison between both groups is shown in Fig. 5G.

3.4 Determining the pathway of cellular inflammation via NF- κ B pathways in MWCNT exposed mice

To investigate whether NF- κ B activation contributes to MWCNT-exposure induced proinflammatory signaling pathways, we used RT-qPCR to analyze transcriptional expression of canonical (TNF- α , IKK- β , I κ B- α , RelA) and alternative pathways (OPG, IKK- α , RelB). We observed an increase in expression IKK- β in MWCNT-exposed animals compared to FA-controls (Figs. 6A, $p < 0.050$). However, no significant changes in I κ B- α were observed between FA-CT and MWCNT-exposed groups (Fig. 6B). Additionally, the average normalized expression of RelA was not statistically altered between MWCNT-exposed vs. FA-CT animals (Fig. 6C). When considering the factors that contribute to alternative NF- κ B activation pathways, expression of OPG was not statistically significant in FA-CT vs. MWCNT-exposed mice (Fig. 6D). However, expression of IKK- α was statistically increased in MWCNT-exposed animals (Fig. 6E, $p < 0.050$). Interestingly, quantification of RelB expression revealed no statistical changes between FA-CT vs. MWCNT-exposed mice (Fig. 6F). Double immunofluorescence microscopy was used to analyze coronary vascular-specific expression of p65/NF- κ B; however, there were no statistically significant differences observed between FA-CT (Figs. 7A-C) vs. MWCNT-exposed mice (Figs. 7D-F). Quantitative comparison between groups shown in Fig. 7G.

3.5 Characterizing the effects of MWCNTs on cholesterol transport

To determine whether inhalation exposure to MWCNTs resulted in altered factors involved in cholesterol transport, LOX-1 and ABCA-1 protein expression were evaluated in the coronary vasculature using double immunofluorescence microscopy. In comparison to FA controls (Figs. 8A-C), we observed a significant increase (~25%) in LOX-1 expression in the coronary vasculature MWCNT exposed mice (Figs. 8D-F, $p < 0.050$); quantitative comparison shown in Fig. 8G. Due to low basal expression of LOX-1 in the healthy vasculature, LOX-1 mRNA transcriptional expression was not able to be analyzed in the ventricular homogenate of the FA control mice. Furthermore, compared to that observed in FA controls (Figs. 9A-C), we noted a significant decrease in coronary vascular ABCA-1 protein activity in MWCNT-exposed animals (Figs. 9D-F, $p < 0.050$); quantification comparison shown in Fig 9G. Interestingly, cardiac ABCA-1 mRNA transcript levels (Fig. 9H) did not show a statistical difference between the FA-CT and MWCNT-groups. This observed difference between the MWCNT-mediated decrease in ABCA-1 protein expression vs. mRNA levels may be due, at least in part, to the use of ventricular homogenate for the RT-qPCR detection vs. quantification of ABCA-1 protein levels localized in the coronary vascular endothelium via double-immunofluorescence.

3.6 Detection of miRNAs involved in heart pathology in MWCNT exposed mice

Due to their established role in cardiovascular homeostasis and/or pathology, we chose to analyze the expression of three key miRNAs, namely miR-1, miR-21, and miR-221 in the hearts of our study animals, via real time RT-qPCR. We observed a significant increase in

cardiac miR-221 expression in mice exposed by inhalation to MWCNT vs. FA controls (Fig. 10A, $p<0.050$), while expression of cardiac miR-21 and miR-1 remained unchanged between the MWCNT and FA exposure groups (Fig. 10B,C).

3.7 Induction of reactive oxygen species in MWCNT-exposed mice.

We used dihydroethidium (DHE) staining as an indicator of superoxide production in the coronary vasculature, as previously described by our laboratory, as well as others (Lund et al., 2009; Ling et al., 2017). Compared to FA controls, statistical analysis using a student's t-test showed ventricular sections from MWCNT-exposed animals showed a significant increase of ROS production, compared to FA-controls (Fig. 11A-C, $p<0.050$). Importantly, the same relationship was also observed for each of the baseline MWCNT vs. FA-control sections (no pre-treatment with an inhibitor control) as well as the inhibitor studies shown in Fig. 11 ($p<0.050$).

In a separate set of experiments, we utilized key inhibitors of ROS in an effort to elucidate possible cellular signaling pathways involved in the observed MWCNT-exposure mediated induction of cardiac ROS. 2-way ANOVA revealed that L-NNA and Apocynin, which are inhibitors of nitric oxide synthase (NOS) and NADPH oxidase (NOX), respectively, had statistically significant differences between the baseline DHE for controls vs. MWCNT compared to those measured in the respective inhibitor studies (e.g. $p<0.050$). Whereas inhibitors such as Allopurinol, BAY-11-7085, and S-DECT, although each generally suppressed overall ROS generation to some degree, had no significant difference when compared between baseline standards vs. inhibitor groups of FA-CT/MWCNTs.

4. Discussion.

There is an increasing number of studies in the literature that report MWCNT-exposure mediating pathophysiological outcomes in the cardiovascular system (Yamashita et al., 2010; Thompson et al., 2014, 2016). For example, MWCNT-exposure has been associated with vascular endothelial cell activation, altered endothelial-dependent arteriolar vasodilation, induction of inflammatory signaling pathways, oxidative stress, lipid peroxidation, and DNA damage (Mandler et al., 2017; Cao et al., 2014); however, the signaling pathways involved have not yet been fully elucidated. Additionally, many of the studies in the literature utilize instillation or aspiration as the route of MWCNT-exposure instead of inhalation, which is more physiologically relevant to human exposure scenarios. As such, we investigated the effects of subacute inhalation exposure to MWCNTs in healthy C57Bl/6 wildtype mice.

Previous studies have reported MWCNT-exposures (notably at much lower doses than used in our subacute study), induce fibroblast differentiation and fibrosis in the lungs via TGF- β 1-mediated signaling pathway (Dong and Ma, 2017). Interestingly, there was no significant induction of inflammatory signaling in the lung (Supplementary Fig. 1) in our studies, which is in agreement with previous studies from animals receiving the same dose/duration of exposure (Mitchell et al., 2007). Furthermore, RT-qPCR analysis on TGF- β 1 was not significantly changed, but this may be due to differences in fibrotic pathways between studies involving lungs vs. the heart cannot be directly compared. However, in an attempt to

quantify alterations of collagen deposition in cardiac tissue; we stained for collagen with Masson's trichrome. We assessed the total cardiac collagen levels compared to the total image area, which did not show any significant alterations across study groups. We then evaluated the same slides/images for overall saturation of collagen staining (e.g. intensity) in cardiac tissue, which revealed that MWCNT-exposed animals exhibited greater saturation compared to controls ($p<0.050$); we believe this is important as increased color saturation translates to greater deposition of collagen in these specific regions. Our findings suggest that modest alterations in collagen may be present in the cardiac tissue, but understanding how this could be occurring mechanistically still remains in question.

Increased oxLDL-LOX-1 signaling is known to result in increased MMP-9 expression and activity, which is associated with progression of atherosclerosis, plaque rupture, platelet aggregation, and other factors involved in CVD (Pirillo et al., 2013). Inhalation exposure to MWCNT resulted in a significant increase in MMP-2/9 activity in the coronary vasculature and increased transcript levels of cardiac MMP-9 in MWCNT-exposed C57Bl/6 animals ($p<0.050$). Findings of MWCNT-exposure inducing MMP-2/9 activity are consistent with that previously reported in the literature, where (aspirated) MWCNT-exposure resulted in significant increases in pulmonary and systemic (serum) MMP-2/9 in C57Bl/6 mice, which also resulted in altered vascular function (Aragon et al., 2016). Such indications, in our study and previous literature, finds that MMP activity and expression is increased in response to MWCNT exposure.

When examining factors associated with vascular injury and proinflammatory mediators, we observed varied results among endpoints analyzed across our study groups. For example, ET-1 was only marginally increased from MWCNT-exposure, which is not in agreement with findings reported from other studies. At least one study has reported an increase in ET-1 (*ex vivo*, coronary effluent) resulting from an acute MWCNT-exposure (i.e., 100 μ g, Thompson et al., 2014). Furthermore, RT-qPCR of VCAM-1 or ICAM-1 did not display significant changes in cardiac expression in our study, despite previous studies reporting a significant increase in expression of both, in addition to ROS induction, in human endothelial cells from MWCNT exposure (Cao et al., 2014). However, it is important to note these previously reported results on MWCNT-mediated alterations in ET-1 and VCAM-1/ICAM-1 were from *ex vivo/in vitro* assays (e.g. Langendorff isolated heart perfusate, endothelial cell culture), and thus cannot be directly compared to the current study endpoints. And while (total) cardiac VCAM-1 mRNA transcript was not statistically altered in our MWCNT vs. FA animals, VCAM-1 protein expression was observed to be statistically elevated in the coronary vasculature of MWCNT-exposed animals, compared to FA-controls ($p<0.050$). This difference in transcript vs. protein expression of MWCNT-mediated induction of VCAM is likely due to the difference in quantifying total cardiac expression (mRNA) vs. coronary vessel-specific expression, where VCAM-1 is predominantly expressed. Previous *in vivo* studies have defined a critical role for VCAM-1 in early lesion formation, due to its rapid induction under inflammatory stimuli such as TNF- α and LOX-1 overexpression (Akhmedov et al., 2014), and subsequent role in mediating monocyte adhesion in the vascular wall. Additionally, investigation of alterations of inflammatory mediators resulting from MWCNT-exposure showed significant induction of TNF- α mRNA levels in cardiac tissue, as well as increased in expression in coronary

vessels ($p < 0.050$). TNF- α is known to be involved with the pathogenesis of several disease states in the cardiovascular system (Feldman et al., 2000). Furthermore, TNF- α has also been reported to be upregulated in the BALF of both wildtype and atherogenic ApoE $^{-/-}$ mice, following MWCNT exposure (i.e., 25.6 $\mu\text{g}/\text{mouse}$ for 5 weeks; Cao et al., 2014), linking roles between MWCNT-exposure and induction of inflammatory signaling molecules. *In vitro* MWCNT-exposure studies have reported induced oxidative stress, alterations in intracellular redox reactions, and activation of NF- κ B signaling cascades in both rat (5 μg , 12/24h) and human (20 $\mu\text{g}/\text{mL}$, 24h) lung cells (Ravichandran et al., 2010; He et al., 2011). However, to our knowledge, the role of inhaled MWCNT-exposure on cardiac NF- κ B signaling cascades, *in vivo*, has not yet been reported. When evaluating factors involved in the canonical and alternative pathways of NF- κ B signaling cascades, it is unclear whether MWCNT-exposure, at this concentration and duration of exposure, results in alterations of cellular expression of p65/p52-NF- κ B complexes. We observe both cardiac IKK- α and β mRNA upregulated in MWCNT-exposed animals vs. FA controls ($p < 0.050$). Both IKK- α and β are capable of ubiquitination and degradation of I κ B- α , following signal transduction pathways, from members of the TNF superfamily such as TNF- α and OPG in vascular disease-states (Galeone et al., 2013). And while cardiac levels of OPG were elevated in our MWCNT animals, we believe that overall low cardiac expression of OPG, and our study n value ($n=7$, $\alpha=0.5$) used for statistical analysis, likely affected overall significance. It is also important to note that although cardiac IKK- α/β was upregulated, I κ B- α was not significantly altered in expression at the transcript level, nor were RelA or RelB. In conjunction with the lack of observable alterations of coronary vascular NF- κ B expression, it is plausible that we may be observing the initial stages of induction of NF- κ B signaling pathways at a 14d time point. However, chronic studies (with multiple time points/durations of exposures), are necessary to further characterize the regulation of key factors such as RelA/RelB and OPG with inhalation MWCNT-exposure.

In an effort to determine whether cholesterol transport may be altered in the coronary vasculature with MWCNT-exposure, we assessed expression of LOX-1 receptor and ABCA-1 transporter protein. LOX-1 has been known to mediate internalization of oxLDL and signal transduction pathways resulting in production of several proatherogenic factors including ROS, ET-1, and MMP-9 expression, in addition to others (rev. in Ogura et al., 2009). We observed a significant increase in LOX-1 receptor expression in the coronary vasculature of MWCNT-exposed animals ($p < 0.050$); and while not a MWCNT-exposure, findings from at least one other study reported similar observations in an *in vitro* model, such that exposure to nanomaterials resulted in increased LOX-1 in macrophages (Niwa and Iwai, 2007). We also observed a significant reduction in coronary ABCA-1 transporter protein, which promotes cholesterol efflux, in MWCNT-exposed C57Bl/6 mice ($p < 0.050$). These findings, altogether, suggest that inhalational MWCNT-exposure can alter cholesterol transport homeostasis, likely from initial inductions of ROS, in healthy animal models.

Understanding the regulatory role of miRNAs, as well as how they contribute to the pathophysiology of CVDs, has been the focus of many recent research and review articles (Feinberg and Moore, 2016; Liu et al., 2010). One *in vitro* study using primary neonatal rat cardiomyocytes found that overexpression of miR-221 may provoke cardiomyocyte hypertrophy (Wang et al., 2012). Furthermore, *in vitro* studies involving human vascular

ECs have reported that miR-221 may serve in either a “protective” or proatherogenic role in the vasculature. For example, miR-221 is initially believed to be upregulated in response to inflammation, by attenuating elevated endothelial NOS activity (Suarez et al., 2015). However, continuous miR-221 upregulation is reported to promote proliferation, migration of vascular ECs, and reduced bioavailability of NO, which are associated with atherosclerosis (Christiakov et al., 2015a,b). In the current study, we observed a significant increase in cardiac miR-221 expression in MWCNT-exposed C57Bl/6 mice, compared to FA controls, which when coupled with increased cardiac TNF- α mRNA suggests that the exposure is likely promoting a proinflammatory state in the heart. Furthermore, miR-21 and miR-1 have been implicated in previous literature to play a role in atherosclerosis and acute myocardial infarction, respectively (Feinberg and Moore, 2016); however, we did not observe any MWCNT-mediated alteration in expression of either in our study.

Previous studies have provided evidence that nanomaterials, including MWCNTs, can cause indirect toxicity at the tissue and cellular level through the generation of ROS and induction of secondary signaling pathways leading to cell-death (Long et al., 2017). Our results show that inhalation MWCNT-exposure results in a significant induction of ROS in the coronary vasculature of C57Bl/6 mice. Furthermore, our 2-way ANOVA analyses on DHE(+inhibitors) vs. DHE(-inhibitors) agree with previous research that reports MWCNTs induce NADPH oxidase-generated ROS, mediating inflammation and fibrosis, in the lung and in human macrophages models (Ye et al., 2011; Sun et al., 2015). Few studies also have also reported endothelial/NOS-related pathways as a primary pathway involved in MWCNT-mediated induction of ROS (Aragon et al., 2016; Lee et al., 2012). The remaining inhibitors, such as S-DECT (SOD inhibitor), BAY-11-7085 (NF- κ B inhibitor), and allopurinol (XO inhibitor) did not show statistically different DHE fluorescence, compared to baseline; suggesting that these pathways are likely not contributing to the induction of ROS in the coronary vasculature of MWCNT-exposed animals in our studies. While these initial studies provide some insight into signaling pathways involved in MWCNT-induced oxidative stress in the coronary vasculature; further studies are required to elucidate the mechanistic pathway(s) involved.

Collectively, our results suggest that MWCNT exposure can induce several key factors involved in inflammation and atherogenesis. While the concentration of MWCNT used in the current study (1 mg/m³ via inhalation) would be considered higher than most occupational levels expected to be encountered in human exposure scenarios, there was notably no significant induction of pulmonary inflammatory signaling pathways observed in the study animals (supplemental data). When comparing the current results with those in the literature, we must consider the MWCNT dose, duration of exposure, route of exposure, and the animal model used. For example, in a study by Han et al., 2015, female atherosclerotic apolipoprotein null (ApoE ^{-/-}) mice were exposed to 40 μ g of MWCNT, once a wk for 16 wks via pharyngeal aspiration, resulting in increased pulmonary inflammation but no discernable change in atherosclerotic lesion formation and/or plasma cholesterol levels. Conversely, MWCNT-exposure (10 or 60 mg/kg) via i.v. injection for 15 or 60 days resulted in hepatotoxicity associated with altered cholesterol biosynthesis (Ji et al., 2009). Muller et al., observed a dose-response related increase in lung inflammation using doses of 0.5, and 2 mg that was intratracheally administered into Sprague-Dawley rats (Muller et al., 2008);

however, these observations were made in lung tissue and BALF collected at just 3 days post MWCNT. Reports from two subchronic studies (60 and 90 days), show both inhaled and i.t. instilled MWCNTs (0.5 – 5mg/animal and 0.1–2 mg/m³, respectively) result in pulmonary inflammation and fibrosis in a healthy animal model (Muller et al., 2005; Ma-Hock et al., 2009). The dose, route of administration, duration of the study protocol, data evaluation, and the models used in each of these studies likely contribute to the difference in pulmonary and cardiovascular endpoints assessed. Additional inhalation studies with a range of doses/duration of exposure (acute and chronic) are necessary to fully characterize MWCNT-mediated toxic responses in the cardiopulmonary system and vasculature.

It is important to note the limitations of the current study. The dosage administered to healthy C57Bl/6 mice (1 mg/m³) is higher than that which would likely be encountered environmentally and/or occupationally in human-exposure scenarios. The National Institute of Health and Occupational Safety (NIOSH) has set a recommended exposure limit (REL) of 1 µg/m³ over an 8 hr TWA for carbon nanotube-exposures; although within the context of the dosage used in the current study (1 mg/m³), it is within the limits of NIOSH exposure guidelines for human general industry occupational exposures for nuisance dust [5 mg/m³ respirable time-weighted average (TWA)] (NIOSH, 2012). Additionally, the 14d exposure time point only gives us insight into what is occurring at that stage of the MWCNT-induced cardiopulmonary response. Because of this, it is plausible that early inflammatory responses from the inhalation exposure to MWCNTs in our study have begun to resolve, and the (sub)chronic exposure-related outcomes not yet manifest, by the day 14 time point. Importantly, using a wildtype animal for these studies allows us to characterize how inhalation MWCNT-exposure may initiate vascular injury, in an otherwise healthy subject, which provides a foundation on which more qualitative assessments can be designed.

5. Conclusion

In conclusion, findings from this study demonstrate that subacute inhalation exposure of MWCNTs results in altered expression of key receptors that mediate cholesterol transport, namely LOX-1 and ABCA-1, within the coronary vascular endothelium of C57Bl/6 wildtype mice. Additionally, inhalation MWCNT-exposure resulted in an increase in cardiac collagen intensity, induction of cardiac transcript expression of TNF- α , MMP-9, and miR-221 expression, which are associated with elevated MMP-2/9 activity, VCAM-1 expression, and ROS production, in the coronary vasculature. Mechanistic studies, using inhibitors of key pathways that are known to contribute to the generation of cellular ROS, showed inhibition of NOS and NOX resulting in significant attenuation of coronary vascular ROS in MWCNT-exposed animals. Furthermore, to understand MWCNT-exposure on canonical and alternative mechanisms in the NF- κ B signaling cascade, our study found increased mRNA expression of cardiac IKK- α/β . These findings suggest that a subacute inhalation exposure to 1 mg/m³ MWCNT promotes proinflammatory signaling and initiates pathophysiological changes in the heart and coronary vascular endothelium, at the cellular and tissue level, which may provide insight into possible mechanistic pathways that contribute to MWCNT-mediated cardiotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations.

CAD	coronary artery disease (CAD)
CNTs	Carbon Nanotubes
COX-2	cyclooxygenase-2
CVD	cardiovascular disease
DHE	dihydroethidium
DQ-gelatin	dye quenched-gelatin
ECM	extracellular matrix
ET-1	endothelin-1
FA-CT	Filtered air control
ICAM-1	intercellular adhesion molecule-1
IκB-α	Inhibitor of κB-α
IKK	Inhibitor of κB Kinase
IL-1β	interleukin-1 beta
IL-8	interleukin-8
LDL	low-density lipoprotein
L-NNA	L-N ^G -Nitroarginine
LOX-1	oxidized low-density lipoprotein receptor-1
MCP-1	monocyte chemoattractant protein-1 (MCP-1)
miRNA	microRNA
miR(-21/-221/-1)	miRNA-21/-221/-1

MMP-9	matrix metalloproteinase-9
mRNA	messenger RNA
MWCNTs	multi-walled carbon nanotubes
NF-κB	nuclear factor- κ -light-chain-enhancer of activated B cells
NOS	nitric oxide synthase
NOX	NADPH Oxidase
ox-LDL	oxidized LDL
RANKL	receptor activator of nuclear factor kappa-B ligand
ROS	reactive oxygen species
RT-qPCR	real-time reverse transcription- quantitative polymerase chain reaction
SOD	superoxide dismutase
S-DECT	sodium diethyldithiocarbamate trihydrate
TNF-α	tumor necrosis factor-alpha
VCAM-1	vascular cell adhesion protein-1
VMSC	vascular smooth muscle cells
vWF	von-Willebrand factor
XO	xanthine oxidase

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Highlights

- Inhaled MWCNT-exposure increases coronary LOX-1 and decreases ABCA-1 expression
- Inhaled MWCNT-exposure amplifies cardiac MMP-2/9 activity and collagen deposition
- Cardiac VCAM-1, TNF- α , and NF- κ B pathway factors increase with MWCNT-exposure
- MWCNT-exposure induces coronary ROS production via NOX and NOS-related pathways
- Cardiac expression of miRNA-221 is elevated with inhalation exposure to MWCNTs

Figure 1. Comparison of fibrosis in coronary arteries of filtered air vs. MWCNT-exposed C57Bl/6 mice.

(A-B) Representative image of Mason's Trichrome staining in cardiac tissue from FA-CT vs. MWCNT- exposed animals, respectively. Red indicates ventricular muscle tissue. Blue indicates collagen fibers. Purple indicates cardiomyocyte nuclei. Imaged at 40 $\times$ magnification. (C) Graph representing statistical analysis of isolated collagen intensity (D) Graph representing statistical analysis of collagen per unit area. * $p < 0.050$ compared to FA-controls

Figure 2. MWCNT-exposure induces MMP-2/9 activity in the coronary vasculature of C57Bl/6 mice.

(A-B) Representative image of *in situ* zymography in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. Green indicates areas of MMP-2/9 expression. Blue indicates cardiomyocyte nuclei. Imaged at 40 $\times$ magnification. Scale bars = 100 μ m. (C) Graph representing average relative fluorescent units (RFUs) of coronary vessels using *in situ* zymography. (D) Graph representing RT-qPCR analysis of MMP-9 mRNA expression normalized to a GAPDH reference gene. * $p < 0.050$ compared to FA-controls.

Figure 3. Expression of biomarkers of coronary artery disease in MWCNT vs. filtered-air exposed C57Bl/6 mice.

RT-qPCR analysis of (A) ICAM-1 mRNA; (B) VCAM-1 mRNA; (C) ET-1 mRNA; (D) TNF- α mRNA; (E) IL-16 mRNA; and (F) IL-1 β mRNA expression between filtered air (FA)-CT and MWCNT-exposed mice. Data reported as mean normalized gene expression to GAPDH reference gene. An n=7 per group was used to analyze each gene of interest.

*p<0.050 compared to FA-controls.

Figure 4. Coronary vessel VCAM-1 expression is increased in C57Bl/6 mice exposed to MWCNT vs. filtered air controls.

(A-C, D-F) Representative image of double immunofluorescence microscopy for VCAM-1 in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. Red indicates areas of VCAM-1 expression. Green indicates vessel endothelial lining. Blue indicates cardiomyocyte nuclei. Imaged at 40 $\times$ magnification. Scale bars = 100 μ m. (G) Graph representing average relative fluorescent units (RFUs) for VCAM-1 activity. * $p < 0.050$ compared to FA-controls.

Figure 5. TNF- α expression is elevated in the coronary vasculature of C57Bl/6 mice exposed to MWCNT vs. filtered air controls.

(A-C, D-F) Representative image of double immunofluorescence microscopy for TNF- α in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. Red indicates areas of TNF- α expression. Green indicates vessel endothelial lining. Blue indicates cardiomyocyte nuclei. Imaged at 40 $\times$ magnification. Scale bars = 100 μ m. (G) Graph representing average relative fluorescent units (RFUs) for TNF- α expression. * $p < 0.050$ compared to FA-controls.

Figure 6. Expression of key factors in canonical and alternative pathways of NF- κ B signaling in cardiac tissue from MWCNT vs. filtered-air exposed C57Bl/6 mice.

RT-qPCR analysis of (A) IKK- β mRNA; (B) I κ B- α mRNA; (C) RelA mRNA; (D) OPG mRNA; (E) IKK- α mRNA; and (F) RelB mRNA expression between filtered air (FA)-CT and MWCNT-exposed mice. Data reported as mean normalized gene expression using GAPDH as the reference gene. An n=7 per group was used to analyze each gene of interest. *p<0.050 compared to FA-controls.

Figure 7. p65/NF- κ B expression is not altered by MWCNT-exposure in the coronary vasculature of C57Bl/6 mice.

(A-C, D-F) Representative image of double immunofluorescence microscopy for p65/NF- κ B in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. Red indicates areas of p65/NF- κ B expression. Green indicates vessel endothelial lining. Blue indicates cardiomyocyte nuclei. Imaged at 40 $\times$ magnification. Scale bars = 100 μ m. (G) Graph representing average relative fluorescent units (RFUs) for p65/NF- κ B expression. * $p < 0.050$ compared to FA-controls.

Figure 8. Coronary vessel LOX-1 expression is significantly increased in C57Bl/6 mice exposed to MWCNT vs. filtered air.

(A-C, D-F) Representative image of double immunofluorescence microscopy for LOX-1 in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. Red indicates areas of LOX-1 expression. Green indicates vessel endothelial lining. Blue indicates cardiomyocyte nuclei. Imaged at 40 $\times$ magnification. Scale bars = 100 μ m. (G) Graph representing average relative fluorescent units (RFUs) for LOX-1 activity. * $p < 0.050$ compared to FA-controls.

Figure 9. MWCNT-exposure results in decreased ABCA-1 expression in the coronary vasculature of C57Bl/6 mice.

(A-C,D-F) Representative image of double immunofluorescence microscopy for ABCA-1 in cardiac tissue FA-CT vs. MWCNT-exposed animals, respectively. Red indicates areas of ABCA-1 expression. Green indicates vessel endothelial lining. Blue indicates cardiomyocyte nuclei. Imaged at 40× magnification. Scale bars = 100 μ m. (G) Graph representing average relative fluorescent units (RFUs) for ABCA-1 activity. (H) Graph representing RT-qPCR scoring of ABCA-1 expression between FA-CT and MWCNT exposures, normalized to GAPDH references gene. * $p < 0.050$ compared to FA-controls.

Figure 10. Cardiac miRNA expression associated with cardiovascular disease in C57Bl/6 mice exposed to MWCNT vs. filtered-air.

RT-qPCR analysis of (A) miR-221 expression; (B) miR-21 expression; and (C) miR-1 expression between FA-CT and MWCNT exposed mice, normalized to SNORD96 housekeeping gene. An n=7 per group was used to analyze each gene of interest. *p<0.050 compared to FA-controls.

Figure 11. Source(s) of coronary artery reactive oxygen species production in MWCNT vs. filtered air exposed C57Bl/6 mice.

(A-B) Representative images of DHE in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. (C) Graph representing mean DHE fluorescence in coronary vessels. (D-E) Representative image of L-NNA (NOS) DHE in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. (F) Graph representing mean DHE fluorescence of L-NNA-treated coronary vessels ($\dagger p < 0.050$). (G-H) Representative image of S-DECT (SOD) DHE in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. (I) Graph representing mean DHE fluorescence of S-DECT-treated coronary vessels. (J-K)

Representative image of BAY-11-7085 (NF- κ B) DHE in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. (L) Graph representing mean DHE fluorescence of BAY-11-7085-treated coronary vessels. (M-N) Representative image of allopurinol (XO) DHE in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. (O) Graph representing mean DHE fluorescence of allopurinol-treated coronary vessels. (P-Q) Representative image of apocynin (NOX) DHE in cardiac tissue from FA-CT vs. MWCNT-exposed animals, respectively. (R) Graph representing mean DHE fluorescence of apocynin-treated coronary vessels ($\dagger p < 0.050$). Red fluorescence = DHE positive staining (ROS). Imaged at 40 $\times$ magnification. Scale bars = 100 μ m. * $p < 0.050$ compared to inhibitor-treated FA-Controls within groups (student's t-test). $\dagger p < 0.050$ reductions in MWCNT-induced DHE +inhibitor, compared to MWCNT-exposed with no inhibitor pre-treatment (2-way ANOVA).