Pulmonary instillation of MWCNT increases lung permeability, decreases gp130 expression in the lungs, and initiates cardiovascular IL-6 transsignaling

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Thompson LC, Holland NA, Snyder RJ, Luo B, Becak DP, Odom JT, Harrison BS, Brown JM, Gowdy KM, Wingard CJ. Pulmonary instillation of MWCNT increases lung permeability, decreases gp130 expression in the lungs, and initiates cardiovascular IL-6 transsignaling. Am J Physiol Lung Cell Mol Physiol 310: L142–L154, 2016. First published November 20, 2015; doi:10.1152/ajplung.00384.2014.—Pulmonary instillation of multiwalled carbon nanotubes (MWCNT) has the potential to promote cardiovascular derangements, but the mechanisms responsible are currently unclear. We hypothesized that exposure to MWCNT would result in increased epithelial barrier permeability by 24 h postexposure and initiate a signaling process involving IL-6/gp130 transsignaling in peripheral vascular tissue. To test this hypothesis we assessed the impact of 1 and 10 μg/cm² MWCNT on transepithelial electrical resistance (TEER) and expression of barrier proteins and cell activation in vitro using normal human bronchial epithelial primary cells. Parallel studies using male Sprague-Dawley rats instilled with 100 μg MWCNT measured bronchoalveolar lavage (BAL) differential cell counts, BAL fluid total protein, and lung water-to-tissue weight ratios 24 h postexposure and quantified serum concentrations of IL-6, soluble IL-6r, and soluble gp130. Aortic sections were examined immunohistochemically for gp130 expression, and gp130 mRNA/protein expression was evaluated in rat lung, heart, and aortic tissue homogenates. Our in vitro findings indicate that 1 μg/cm² MWCNT decreased the development of TEER and zona occludens-1 expression relative to the vehicle. In rats MWCNT decreased pulmonary vascular permeability and can result in adverse cardiovascular/systemic responses (39, 56). Various forms of nano-sized particles have been shown to alter lung permeability similar to acute lung injury models (5, 20, 33, 52, 71). Although MWCNT exposure has caused notable inflammatory responses in an alveolar-capillary cell culture model (53), in vivo changes in lung permeability caused by MWCNT have not been well documented.

Lung permeability at the alveolocapillary barrier is regulated by tight junctions between alveolar epithelial cells and capillary endothelial cells. The barrier regulates the net movement of fluid, solutes, and circulating cells between the bloodstream, interstitium, and alveolar space (48). Several cell signaling cascades and proteins function to maintain the barrier’s integrity through manipulation of the cytoskeleton, which maintains cell shape, cell-cell interactions, and cell adhesions with the extracellular matrix (28). In response to inflammatory signals, some of these cascades and proteins function to rearrange the cytoskeleton to alter cell shape, loosen cell-cell contact, and break connections with the extracellular matrix in an effort to increase barrier permeability (48). In response, the alveolocapillary barrier becomes leaky, promoting the diffusion of inflammatory mediators and chemoattractants into adjacent compartments, allowing for the recruitment of circulating leukocytes, and phagocytosis and antigen-presenting cells to migrate into the lymphatics (28). Since pulmonary exposure to MWCNT can result in an interaction of MWCNT and the epithelial cells of the lung (52), we were interested in evaluating how MWCNT modify the integrity of the pulmonary epithelium leading to the translocation of mediators outside of the lung.

We postulated that changes in glycoprotein 130 (gp130) expression could serve as a marker of MWCNT exposure and may link any disrupted bronchial epithelial barrier to the resultant derangements.

MULTIWALLED CARBON NANOTUBES (MWCNT) are being utilized in commercial products throughout the medical, military, automotive, aerospace, and consumer industries. The expanding applications for MWCNT increase the probability of human exposure via inhalation during manufacturing processes.

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cardiovascular detriments associated with MWCNT exposure. The proposed mechanism is based on the links between MWCNT exposure and interleukin-6 (IL-6) and nuclear factor-kappa B (NF-κB) activation (14, 15). Gp130 is a transmembrane signal transducer for IL-6, which has been linked to canonical activation of NF-κB (11, 13). We have previously reported that cyclooxygenase-2 (COX-2) may be involved in vascular impairments 24 h after 100 μg MWCNT instillation (59) and that NF-κB has been linked to the induction of COX-2 in vascular endothelial cells (34, 50). Together these reports suggest that MWCNT exposure could be linked to COX-2 in cardiovascular tissue via IL-6 and NF-κB. However, most cells that comprise pulmonary and cardiovascular tissues lack a membrane-tethered IL-6 receptor alpha (IL6α), requiring the presence of soluble IL6α (sIL6r) to allow IL-6 activation of gp130, a nontraditional signaling protein termed IL-6 transsignaling. This mechanism may be regulated antagonistically by the presence of a soluble form of gp130 (sgp130) (2). When IL-6/sIL6r complex is in excess of sgp130, the IL-6/sIL6r complexes can bind transmembrane gp130 at the cell surface. Once bound, transmembrane gp130 activates Janus kinase/signal transducer and activator of transcription (JAK/STAT) to drive intracellular responses (16). IL-6 transsignaling has been shown to upregulate gp130 expression in cellular vessels (22, 45). Binding to gp130 has been shown to induce NF-κB-mediated responses in vitro (11). We postulate a role of IL-6 transsignaling and increasing gp130/NF-κB/COX-2 expression patterns and promotion of systemic inflammation following MWCNT exposure that may establish a link to the cardiovascular derangements we have previously reported (29, 59, 64).

To date it is unknown what the molecular mechanism linking the pulmonary responses to MWCNT exposure with detrimental cardiovascular end points. We hypothesized that MWCNT increases alveolar permeability and initiates IL-6 transsignaling. We tested this hypothesis by 1) exposing airway epithelial cells to MWCNT in vitro and 2) assessing elements of the signaling pathway following pulmonary instillation of MWCNT in rats. The primary end points examined were lung/airway permeability, serum concentrations of IL-6 transsignaling agents, and evidence of altered IL-6 transsignaling in cardiovascular tissue.

MATERIALS AND METHODS

MWCNT suspensions. Vehicle instillate was comprised of 10% surfactant/saline by diluting Infasurf calf surfactant (ONY, Amherst, NY) to 10% with sterile normal saline. Infasurf is comprised of clinical grade phospholipids, neutral lipids, and surfactant-associated proteins B and C. MWCNT, provided by NanoTechLabs (Yadkinville, NC), were suspended in the vehicle suspension at 0.5 mg/ml and sonicated (25 nm, with a 113.103 m2/g surface area and 0.688 cm3/g pore volume. In suspension the hydrodynamic size also showed bimodal distribution with peaks at 200 ± 50 and 1,000 ± 150 nm, the zeta potential was 44.6 mV, and the isoelectric pH was 3.5.

Cell culture and TEER. Normal human bronchial epithelial primary cells (NHBE) and growth media were obtained from Lonza (Allendale, NJ) in BEGM BulletKits at passage 2 (Lot no. 000295568) and seeded at 1.5 × 104 cells/cm² in eight-chamber gold-electrode E10+ arrays (Applied Biophysics, Troy, NY). Arrays were precoated with a collagen solution (30 μg/ml collagen 1A1, 10 μg/ml fibronectin, 10 μg/ml bovine serum albumin in sterile saline) and allowed to dry prior to UV sterilization. Cells were incubated at 37°C in 5% CO2 and BEGM growth media was refreshed every 2 days. NHBE cultures were grown to confluent monolayers before being treated with media alone, media plus 10% Infasurf (vehicle), vehicle plus 1 μg/cm² MWCNT, or vehicle plus 10 μg/cm² MWCNT. Separate wells containing treatment suspensions in the absence of cells were also measured to control for potential electrical interference by the vehicle or MWCNT suspensions alone. Transepithelial electrical resistance (TEER) across exposed NHBE monolayers was measured on an electric cell-substrate impedance sensing (ECIS) instrument (1600R model, Applied Biophysics). ECIS takes resistance measurements at 4,000 Hz continuously, providing a kinetic measurement of resistance in ohms over time. TEER was measured for 24 h prior to nanotube exposure to establish a baseline resistance, and then for 24 h postexposure. Measurements were taken under normal incubator conditions (37°C and 5% CO2) in six replicates per treatment. Changing of media routinely causes aberrant fluctuations in TEER measurements for 1–2 h until cells reequilibrate, thus TEER was not reported from 0–2 h postexposure to experimental media.

In-Cell Western Assay. NHBE were seeded in Costar black clear-bottom 96-well plates (Corning 3606) at a seeding density of ~14 × 103 cell per well and culture to >80% monolayer confluence. Samples were run in triplicate. Cells were treated with media, 10% Infasurf (vehicle) surfactant in media or MWCNT with 10% Infasurf at 1 or 10 μg/cm² for 24 h. In-Cell Western Assay (LICOR Biosciences, Lincoln, NE) was performed following 24 h in vitro exposure to MWCNT/Infasurf or Infasurf to assess the changes in target protein expression (66). Briefly, the treated cells were immediately fixed with 3.7% formaldehyde with 1% BSA, permeabilized with 0.1% Triton-X-100, blocked with Odyssey blocking buffer (LICOR Biosciences), and treated with polyclonal antibodies of 1:1,000, cell adhesion molecule 1 (ICAM-1; 1:50), vascular cell adhesion protein 1 (VCAM-1; 1:100) primary antibodies (Abcam, Cambridge, MA). IRDye 800CW Secondary Antibodies (LICOR Biosciences) were used in 1:800 dilution for target proteins and DNA were stained with CellMask Deep Red reagent (ThermoFisher) for cell number normalization. The fluorescence was detected, quantified, and analyzed by use of LICOR Odyssey G Infrared Imaging System and software.

RNA isolation and qPCR from NHBE cells. RNA was isolated from NHBE cells after treatment by RNEasy kit (Qiagen). Complementary DNAs (cDNA) were generated from 1.0 μg of purified RNA by using TaqMan reverse transcription reagents from Applied Biosystems ( Foster City, CA). Real-time PCR was performed with Taqman PCR Mix (Applied Biosystems) in the HT7900 ABI sequence Detection System (Applied Biosystems). Predesigned primers for ZO-1 (Hs01551861), ICAM-1 (Hs00164932), VCAM-1 (Hs01003372), IL-8 (Hs0174103), IL-6 (Hs00985639), and gp130 (Hs01743640) were purchased from Applied Biosystems. Gene expression was normalized to 18s RNA (Hs03003631) and expression levels in untreated samples were set as one utilizing the relative quantification calculation (2−ΔΔCt). Samples were run in triplicate.

Animals. Sprague-Dawley rats were purchased from Charles River at 8–12 wk old, ranging from 200 to 225 g. Rats were housed in the Department of Comparative Medicine at East Carolina University and allowed 1 wk to acclimate before experiments started. Rats were housed in temperature-controlled (25 ± 1°C) units with 12-h light-dark cycles and had access to standard laboratory chow and water ad libitum. All animal use complied with the guidelines of and was approved by East Carolina University’s Institutional Animal Care and Use Committee.

Intratracheal instillation. Rats were anesthetized with 50% isoflurane in propylene glycol. The tongue was briefly exteriorized to expose the trachea. A 200-μl droplet of either MWCNT (100 μg) or vehicle suspension was placed into the opening of the trachea. Rats were monitored for 24 h to ensure their status and placed in athermostatic chambers to ensure their comfort.
of the trachea until completely aspirated. Animals were monitored until normal grooming habits resumed and were returned to standard housing units for the following 24 h.

Pulmonary injury assessment and tissue collection. Twenty-four hours after instillation rats were anesthetized with an intraperitoneal injection of ketamine/xylazine (90/10 mg/kg, respectively). After plane-3 anesthesia was achieved, a pneumothorax was induced and rats were exsanguinated by transecting the inferior vena cava. One cohort of rats had the right lung used for bronchoalveolar lavage (BAL) studies while the left lung was fixed for histological analysis. A second cohort of rats had the right lung used for protein/mRNA expression while the left lung was used for water-to-tissue weight assessment. Heart and thoracic aortic segments were also collected for immunohistochemistry and protein/mRNA expression analysis.

BAL and differential cell counts. The ventral portion of the thoracic cage was removed and a tracheotomy was performed. An 18-gauge angiocatheter was inserted into the trachea, the bronchus of the left lung was clamped at the hilum, and the right lung of each animal was lavaged in situ. Four lavages were performed per animal with ice-cold HBSS, using 26.25 ml/kg body wt. All four lavage samples were centrifuged at 1,000 g for 10 min at 4°C and the pellets were pooled to determine total cell counts by using a Cellometer (Nexcelon Biosciences, Lawrence, MA). Utilizing a CytoSpin IV (Shandon Scientific, Cheshire, UK), we centrifuged 20,000 cells per slide and stained them with a three-step hematoxylin staining kit (Richard-Allan Scientific, Kalamazoo, MI). Cell differential counts were determined by evaluating 300 cells per slide based on morphology to establish a cellular profile, using a light microscope (Jenco International, Portland, OR). The percentage of each individual cell type per slide was multiplied by the total cell counts from each animal for data reporting.

BALF protein concentrations. BAL fluid (BALF) was analyzed for total protein concentration as a readout of lung permeability. Protein concentrations were determined using a Bio-Rad DC Microplate Protein Assay Kit (Bio-Rad, Hercules, CA), per instructions provided by the manufacturer. BALF samples were plated in duplicate on a 96-well plate, read with a BioTek Plate Reader, and analyzed with Gen5 software (BioTek, Winooski, VT).

Lung water/tissue weight. After excision, the left lung was lightly blotted to remove any surface material following the tissue harvest. The lung was immediately weighed (raw weight), then oven-dried in a drying oven at 50°C for 48 h, and then reweighed for dry weight determination. The difference in the weights was used to estimate lung water content.

Left lung histology. Unlavaged left lungs were infused with 10% neutral buffered formalin and fixed at room temperature for 24–72 h. Fixed lungs were processed, embedded in paraffin, sectioned at 5 μm, mounted on slides, and stained with hematoxylin and eosin. Slides were examined with a Leica DM5000 B upright light microscope (Buffalo Grove, IL), Leica DFC 420 color camera, and LASC microscope software.

Serum biochemical analysis. Serum IL-6, sIL-1r, and sgp130 concentrations were measured by commercially available ELISAs. Whole blood was drawn from the right ventricle, 24 h after MWCNT or vehicle instillation, placed in serum separator tubes, and centrifuged at 20,800 g for 30 min at 4°C. Serum was then transferred into clean cryo tubes, frozen in liquid nitrogen, and stored at −80°C. At the time of analysis, serum was thawed and analyzed for IL-6 with a kit from EMD Millipore (no. EZRIL6, Billerica, MA); sIL-1r with a kit from MyBioSource (no. MBS260742, San Diego, CA); and sgp130 with a kit from MyBioSource (no. MBS267808). The ELISAs were performed in flat-bottom 96-well plates according to the manufacturer’s instructions. The optical densities of all wells were measured at 405 nm by use of a Biotek Synergy HT plate reader and analyzed with Gen5 software (Biotek).

Immunohistochemistry. Sections of aorta were mounted on slides, hydrated, and immunostained for gp130 with a polyclonal sheep IgG antibody diluted 1:10 (no. AF5029, R&D Systems, Minneapolis, MN) and an anti-sheep horseradish peroxidase 3,3’-diaminobenzidine (DAB) staining kit (no. CTS019, R&D Systems) per the manufacturer’s instructions. During the primary antibody incubation step some slides were incubated with PBS without the primary antibody as a negative control, containing (in mM) 137 NaCl, 2.7 KCl, 4.3 Na2HPO4 × 7H2O, and 1.47 KH2PO4, pH = 7.4. Each slide was lightly counterstained with Harris hematoxylin, dehydrated with ethanol, and coverslipped. Slides were examined with a Leica DM5000 B upright light microscope, Leica DFC 420 color camera, and LAS microscope software.

Tissue real-time PCR analysis. Right lung, heart, and aortic tissues from rats were homogenized in TRIZol with a bead homogenizer for RNA extraction. RNA was isolated by using a Qiagen RNEasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed by using a QuantiTect Reverse Transcription Kit (Qiagen) according the manufacturer’s directions. A Bio-Rad iCycler IQ, QuantiTect Primer Assays, and SYBR Green Master Mix were used to evaluate expression of gp130 (Rn_Il6st_2_SG), NF-κB (Rn_NFkb1_2_SG), and Cox-2 (Rn_Pigs2_1_SG). Target cDNA levels were normalized to the internal reference Gapdh (Rn_Gapd_1_SG), by using the expression 2−ΔΔCt, where ΔCt is defined as target Ct – internal reference Ct. Four independent experiments were conducted and averaged to derive the reported values.

Western blot. Lung, heart, and aortic tissue segments were homogenized in modified RIPA buffer with a sanded glass mortor and pestle set on ice (1 mg tissue/100 μl buffer). The modified RIPA buffer contained (per 100 ml) 790 mg Tris Base, 90 mg NaCl, pH to 7.4 with 6 N HCl, 1 ml 10% Nonidet P-40, 1 ml 100 mM EDTA, 1 ml protease, and 1 ml phosphatase inhibitors added at the time of homogenization (no. P8340 and no. P0044, respectively, Sigma-Aldrich, St. Louis, MO), and brought to volume with distilled H2O. Tissue homogenates were centrifuged at 20,800 rcf for 20 min. Supernatants were analyzed for total protein content by using a Bio-Rad DC Microplate Protein Assay Kit (Bio-Rad) per the manufacturer’s instructions. Supernatant samples were plated in duplicate on a 96-well plate, read with a Biotek Plate Reader, and analyzed with Gen5 software (BioTek). Supernatant samples were then loaded into wells of a 15-well Mini-Protein TGX, 4–20% mini gel (no. 4561096, Bio-Rad) by loading 25 μg of total protein for heart and lung samples. The supernatant from aortic samples was so dilute that 15 μl were loaded in each well to achieve maximum protein load. Recombinant rat gp130/Fc chimera (2 ng, ~130 kDa, no. 5029-RG, R&D Systems) was loaded into one well as a positive control. After electrophoresis, separated proteins were transferred from gels to Trans-Blot Turbo Mini Nitrocellulose Trans-Blot Pack membranes (no. 170-4158) by use of a Trans-Blot Turbo Mini Transfer Module (Bio-Rad, Hercules, CA), set on ice (1 mg tissue/100 ml). The Trans-Blot Turbo Mini Transfer Module was set on ice (1 mg tissue/100 ml) and run for 1 h at a maximum protein load. Recombinant rat gp130/Fc chimera (2 ng) was loaded in each well to achieve maximum protein load.

Tissue homogenates were centrifuged at 20,800 rcf for 20 min. Supernatants were analyzed for total protein content by using a Bio-Rad DC Microplate Protein Assay Kit (Bio-Rad) per the manufacturer’s instructions. Supernatant samples were then loaded into wells of a 15-well Mini-Protein TGX, 4–20% mini gel (no. 4561096, Bio-Rad) by loading 25 μg of total protein for heart and lung samples. The supernatant from aortic samples was so dilute that 15 μl were loaded in each well to achieve maximum protein load. Recombinant rat gp130/Fc chimera (2 ng, ~130 kDa, no. 5029-RG, R&D Systems) was loaded into one well as a positive control. After electrophoresis, separated proteins were transferred from gels to Trans-Blot Turbo Mini Nitrocellulose Trans-Blot Pack membranes (no. 170-4158) by use of a Trans-Blot Turbo Transfer System (Bio-Rad). Immunostaining for 1) gp130 was done with a polyclonal sheep IgG antibody (no. AF5029, R&D Systems); 2) NF-κB was done with a monoclonal rabbit antibody (no. 4764, Cell Signaling Technology, Danvers, MA); 3) Cox-2 was done with a monoclonal rabbit antibody (Cell Signaling Technology); and 4) Gapdh was done with a monoclonal mouse IgM antibody (no. G8795, Sigma-Aldrich), with a 1:1,000 dilution. Primary antibodies were probed with LICOR infrared (IR) secondary antibodies: donkey anti-goat 800 (no. 926-32214), donkey anti-mouse 700 (no. 926-68022), and donkey anti-rabbit 680RD (no. 926-68073), with a 1:25,000 dilution. Blots were read with an Odyssey CLx (LICOR) and analyzed with Image Studio software (LICOR). Blots were probed for two targets and then stripped and reprobed for the remaining two targets. This was repeated on three separate blots for each tissue. Band intensities were normalized to the Gapdh band from the same blot and MWCNT group ratios were reported as fold change from vehicle. Multiple banding patterns from 90 to 130 kDa were included in our analysis of ex vivo gp130 from tissue homogenates because of the possible multiple posttranslational conditions that influence the molecular weight of gp130 (67).

Statistics. Data are reported as means ± SE. TEER measurements (Fig. 1A) were analyzed by repeated-measures ANOVA with a Bonferroni posttest (not statistically different) and nonlinear regression
analysis. Statistical differences indicated by nonlinear regression analysis were detected by using both a second-order polynomial regression and a segmental linear regression with a constrained intersection at the 10-h time point. Figure 1, B–D were analyzed by ANOVA and linear-trend analysis (not statistically different). Results from rat tissue studies, for serum IL-6, sIL6r, and sgp130 data set a one-way ANOVA was employed with a Dunnett’s posttest vs. Vehicle. All other data were analyzed with t-tests to determine statistical significance between vehicle and MWCNT groups. All differences were considered to be statistically significant at $P < 0.05$.

RESULTS

**TEER in vitro.** TEER tracings for the last 24 h of recordings plus experimental protocol are provided, including positive and negative control groups (Fig. 1). Wells containing no cells but with the 10% surfactant/saline and 10 μg/cm² MWCNT measured consistently between 200 and 300 Ω, regardless of treatment (without cells), verifying that the nanomaterials and Infasurf did not, by themselves, alter the resistance of the culture medium. The largest dose of 10 μg/cm² MWCNT significantly altered the TEER in NHBE monolayers from those of vehicle-treated controls and those treated with 1 μg/cm² MWCNT (Fig. 1, A and B). Differences in the progression of electrical resistance changes ($\Delta$TEER) were determined by two separate nonlinear regression analyses. The 1 μg/cm² MWCNT dose did not alter changes in TEER compared with vehicle over 24 h. The largest differences in TEER between time points were detected at ~16 h postexposure (Fig. 1B). TEER of NHBE cell monolayers treated with 10 μg/cm² MWCNT was on average 35% lower than NHBE treated with vehicle and on average 27% lower than NHBE monolayer treated with 1 μg/cm² MWCNT. We have presented the differences in slope 1 (Fig. 1C) and slope 2 (Fig. 1D) that were derived by segmental linear regressions used to analyze the TEER progression presented in Fig. 1A.

**ZO-1, ICAM, and VCAM protein expression in NHBE cells.** The protein expression of tight junction protein 1 (ZO-1) and VCAM-1 revealed a small but nonsignificant trend for reduction following 24-h treatment with 1 and 10 μg/cm² MWCNT compared with vehicle treated (Fig. 2, B and C). However, the ICAM-1 protein expression was unchanged 24 h after MWCNT exposure as assessed by the In-Cell Western Assay (Fig. 2C).

**Bronchiolar epithelial cell mRNA expression of tight junction proteins, adhesion molecules, and cytokine production.** The influence of MWCNT instillation on circulating levels of lung epithelial barrier integrity, adhesion molecules, and cytokine production is presented in Fig. 3. ZO-1 was increased with 24 h exposure to 1 and 10 μg/cm² MWCNT compared with media or vehicle control (Fig. 3A). ICAM-1 expression was only enhanced with the 10 μg/cm² MWCNT dose (Fig. 3B).
whereas VCAM-1 was suppressed compared with media- and vehicle-treated cells (Fig. 3C). Interleukin 8 (IL-8) expression was enhanced with vehicle exposure but was further augmented with addition of 1 or 10 μg/cm² H9262 (Fig. 3D). IL-6 expression in bronchial epithelial cells was also increased with vehicle treatment but was decreased with exposure to either dose of MWCNT (Fig. 3E). However, gp130 expression was increased with both 1 and 10 μg/cm² MWCNT exposure compared with controls (Fig. 3F).

Lung cellularity and markers of lung permeability. The impact of MWCNT instillation on BAL cell counts and markers of edema from rat lungs 24 h following exposure are presented in Fig. 4. Determination of differential cell counts from BAL collected from MWCNT-instilled rats had nearly a 10-fold increase in eosinophils compared with the number of eosinophils seen in the vehicle group (Fig. 4A). Protein concentrations in BAL fluid collected from MWCNT-instilled rats was 29% greater than protein concentration in BAL fluid collected from vehicle-instilled rats (Fig. 4B). Rat lungs instilled with MWCNT had 47% more lung water per gram of tissue than were measured in rat lungs instilled with (Fig. 4C).

Pulmonary histology-hematoxylin and eosin staining. Representative images from histological analysis of lung tissue collected 24 h following instillation of MWCNT or vehicle are provided in Fig. 5. Lung sections from rats instilled with vehicle showed little to no evidence of wall thickening or cellularity (Fig. 3A). Lung sections from rats instilled with MWCNT showed evidence of wall thickening and decreased alveolar space (Fig. 5B).

Serum IL-6, sIL6r, and sgp130 concentrations. The influence of MWCNT instillation on circulating levels of IL-6 transsignaling agents is presented in Fig. 6. We sought to find evidence for changes in the circulating concentrations of IL-6, sIL6r, or sgp130 consistent with IL-6 transsignaling following MWCNT instillation. Serum IL-6 was detected at >100 pg/ml in serum collected from rats 24 h following exposure to MWCNTs and was below detection in serum collected from vehicle-exposed rats. The concentration of sIL6r was detected at >1,000 pg/ml in serum collected from MWCNT rats but was undetectable in vehicle control serum. Serum levels of sgp130 was undetectable in MWCNT-exposed rats and was >1,000 pg/ml in serum collected from the vehicle group. We have also included serum concentrations of IL-6, sIL6r, and sgp130 from naive rats as controls for the ELISA.

Immunohistochemistry for aortic gp130. The result of MWCNT instillation on aortic gp130 protein expression is illustrated in Fig. 7. Immunohistochemistry has been used to demonstrate changes in gp130 expression in rat aorta (18).
Aortic sections collected from rats instilled with vehicle showed moderate expression of gp130 in the adventitia and smooth muscle layers but the endothelial layer was absent of any staining (Fig. 7A, arrow). This was contrasted by a staining suggestive of expression of gp130 on the endothelial lining of aortic cross sections collected from rats instilled with MWCNT (Fig. 7B, arrow). We provided images of negative controls that were not incubated with anti-gp130 primary antibody in the vehicle (Fig. 7C) and MWCNT groups (Fig. 7D).

mRNA and protein expression of gp130, NF-κB, and Cox-2. The impact of MWCNT instillation on rat lung, heart, and aorta mRNA and protein expression profiles for gp130, NF-κB, and Cox-2 are presented in Fig. 8. We performed these experiments to explore the relationship between gp130 expression and NF-κB/Cox-2 expression in cardiopulmonary tissues. According to RT-PCR experiments, gp130 mRNA expression in lung tissue homogenates was downregulated by twofold, unchanged in the heart tissue homogenates, and upregulated by 2.5-fold in aortic tissue homogenate from rats previously instilled with 100 μg MWCNT compared with the expression in tissues collected from rats instilled with vehicle (Fig. 8A). According to Western blot experiments, gp130 protein expression was reduced in lung tissue homogenates, unchanged in heart homogenates, and unchanged in aortic tissue homogenates in the MWCNT group compared with the vehicle group (Figs. 8B and 9). We provided a representative image of Western blot for gp130 from lung tissue homogenates in Fig. 9. Using RT-PCR we found that Nfκb mRNA expression was unchanged in lung tissue homogenates, downregulated by 3.1-fold in heart tissue homogenates, and upregulated by 2.3-fold in aortic tissue homogenates collected from rats instilled with MWCNT compared with tissue collected from rats instilled with vehicle (Fig. 8C). Protein expression for NF-κB p65, an active subunit of NF-κB, was not significantly different between the MWCNT and vehicle groups in rat lung, heart, and aortic tissue homogenates when analyzed by Western blot (Fig. 8D). According to RT-PCR experiments, COX-2 mRNA expression was unchanged in lung tissue homogenates, downregulated by 5.6-fold in heart tissue homogenates, and unchanged in aortic tissue homogenates collected from rats instilled with MWCNT compared with tissue collected from rats instilled with vehicle (Fig. 8E). Protein expression for COX-2 was not significantly
different between the MWCNT and vehicle groups in rat lung, heart, and aortic tissue homogenates when analyzed by Western blot (Fig. 8F).

DISCUSSION

We have demonstrated that exposure to MWCNT at the pulmonary interface can 1) decrease TEER within 24 h in vitro, 2) promote pulmonary eosinophilia and increase BAL protein and lung water 24 h after exposure in vivo, and 3) induce agents of IL-6 transsignaling in the cardiovascular system 24 h after exposure in vivo. The toxicological and biomedical communities are well aware of the connection between cardiovascular injury and inhaled pollutants, including particulate matter and diesel particles (6, 7, 9, 10, 25, 65). Based on those associations, adverse cardiovascular end points have also been reported following pulmonary exposure to engineered nanomaterials (23, 26, 32, 55, 59, 60, 64). Although many local tissue injury mechanisms have been identified, mechanisms that link pulmonary exposures and cardiovascular end points are not well understood. We proposed that exposure to MWCNT could increase epithelial permeability and promote systemic IL-6 transsignaling, which exerts a negative impact on cardiovascular tissue via IL-6, NF-κB and COX-2 signaling.

Results from in vitro cultures of human primary NHBE cells revealed decreasing electrical resistance, a measure of barrier integrity, with increasing MWCNT concentration, supporting our hypothesis and further supporting another recent study on...
the effect of MWCNT exposure on TEER (44, 52). The TEER of NHBE monolayers exposed to 10 μg/cm² MWCNTs for 24 h was significantly reduced compared with cells exposed to Infasurf alone. However, although the data obtained from the TEER measurements do appear to support our in vivo findings of increased water weight and BALF protein concentration, the Infasurf surfactant vehicle had an unexpected elevating effect on NHBE electrical resistance on its own. NHBE cells treated with 10% Infasurf had TEER measurements two- to fourfold higher than cells treated with only growth medium; however, tight junction protein ZO-1 expression and production was unaltered. This cellular response was not observed in wells containing 10% Infasurf and 10 μg/cm² MWCNT without cells. Use of different pulmonary surfactants has been reported to influence MWCNT-induced inflammation in vitro (12). The cellular response to Infasurf complicates the interpretation of the ECIS instrument results because the MWCNTs consistently reduced monolayer TEER from Infasurf-elevated level; we believe it is reasonable to conclude that MWCNT are capable of reducing TEER in human bronchial epithelial cells when exposed across a 24-h period. Tight junction elements are linked to actin cytoskeleton by the protein ZO-1 and are postulated to be essential for stabilizing tight junctions (63). We observed an impairment of TEER with exposure to MWCNT, in agreement with a recent report by Rotoli et al. (44) with an additional novel finding of an acute loss of proteins staining for ZO-1 and elevation in its mRNA that suggests epithelial cell junction integrity may be disrupted by MWCNT exposure. Although these are novel data following MWCNT exposure, it is a finding that corroborates conclusions from numerous other studies describing disruption of epithelial cells tight junction formation following exposure to cigarette smoke (36, 49), particulate matter (5), or cadmium (3). However, the data presented here indicate that exposure to MWCNT can initially alter barrier function, which may influence tight junction protein expression. Future studies should evaluate the time course of tight junction proteins expression in the airways following MWCNT exposure.

We speculate that reduced ZO-1 expression may be solely responsible for the underlying reduced TEER measurements at the high concentration of MWCNT, similar to the results seen by West et al. (70) using an antibody distribution of the E-cadherin. However, the literature suggests that interactions between the claudins and occludins are critical for the tight junction polarization and establishment of barrier integrity (30). Recent work in both epithelial and endothelial cell cultures suggest that posttranslational modification of the tight junction proteins can modulate that barrier integrity (3, 27, 49, 68). Interestingly, a recent paper by Rochfort and Cummins (40) reported that the cytokine IL-6 could dose dependently reduce the expression of ZO-1 in a microvascular endothelial cell culture. It is currently hypothesized that neither endothelial or epithelial cells express the IL-6 receptor, but these in vivo findings with increased lung water would support the hypothesis that a mechanism of action for the cytokine effect on barrier integrity would be through soluble IL-6 transsignaling process.

Our results show that eosinophilia occurred in response to in vivo pulmonary exposure to MWCNT. Eosinophilia has been documented 24 h following pulmonary exposure to C60 fullerenes (60), zinc oxide nanoparticles (8), as well as MWCNT (17, 35, 41, 46). The eosinophilia we have observed indicates a local inflammatory response in the lung following exposure to MWCNT. MWCNT exposure to bronchiolar epithelial cells upregulated ICAM-1, IL-8, and gp130 expression; however, ICAM-1 protein expression was unaltered at this time point. ICAM-1 (CD54) is a key adhesion molecule in the accumulation of inflammatory cells, including neutrophils, eosinophils, and T lymphocytes in the lung (57, 61, 69). We expected that alterations in this adhesion molecule may be partially responsible for the increase in eosinophils seen in vivo after MWCNT exposure, but the expression is labile and we may not have detected its peak at a 24 postexposure time point. Increases in eosinophils have been noted in other models of MWCNT-exacerbated pulmonary disease (58). Interestingly, VCAM-1 expression and production by NHBEs were decreased with
MWCNT; however, no suppression of inflammatory cells into the air space was noted in vivo. VCAM-1 is constitutively expressed on both respiratory epithelial cell lines and primary bronchial epithelial cells, and expression is induced by translocation of transcription factors NF-κB and GATA to the nucleus (38). The decrease in VCAM-1 expression may suggest that MWCNT alters the translocation of inflammatory cells from the vasculature, a finding that was not noted in our study but has been reported with MWCNT and allergen dosing (51, 58). Taken together, these results indicate that MWCNTs can alter adhesion molecule expression on the epithelium of the lung which can change the inflammatory cell profile in pulmonary tissue. In vitro TEER data together with the BAL protein and lung water data obtained from rats exposed to MWCNT provide support for the hypothesis that local inflammatory conditions in the lung combined with alterations of the pulmonary epithelial barrier may allow inflammatory cells, cytokines, and soluble receptors to reach the systemic circulation and impact cardiovascular tissues. To date, eosinophils have not been linked directly to IL-6 transsignaling but eosinophils contain intracellular stores of IL-6 (24) and IL6r (54). A recent study demonstrated blockade of IL-6r utilizing a monoclonal antibody was able to attenuate eosinophilic lung infiltration in a cockroach-induced allergen model of asthma, but not in a house dust mite model (62), strengthening the link between pulmonary inflammatory responses and IL-6 transsignaling. In the context of our study it seems plausible that eosinophils could play a role as a source of IL-6 transsignaling events.

IL-6 transsignaling is a critical mechanism for IL-6 to yield a response in cardiovascular tissue as normally cells in the cardiovascular system lack expression of membrane tethered IL6Rα, necessary for classic IL-6 signaling (42). An increase in IL-6 concentration alone is not enough to initiate IL-6 transsignaling. Therefore, it is important to consider that MWCNT exposure alters adhesion molecule expression on the epithelium, which could facilitate the systemic impact of IL-6 transsignaling. The data presented in Fig. 8 provide evidence for this hypothesis, showing changes in the expression of gp130, NF-κB, and COX-2 in rat tissues following MWCNT exposure. These changes could facilitate the systemic impact of IL-6 transsignaling, potentially leading to cardiovascular complications.
transsignaling because sgp130 circulates at concentrations that antagonize the activation of membrane gp130 via IL-6/sIL6r complex (19). A complementary rise in circulating IL-6 and sIL6R concentrations should increase the probability of membrane gp130 activation, but a reduction in circulating sgp130 may also be sufficient. Our evidence suggests this novel signaling process may underlie the systemic effects of pulmonary exposure to MWCNT. We find confidence in the ELISAs utilized in this study based on three particular results. First, analysis of serum from naive rats demonstrated that IL-6, sIL6r, and sgp130 were detected at relative concentrations of sgp130 > sIL6r > IL-6, thus preventing IL-6 signaling (31). Second, analysis of serum collected from rats instilled with vehicle is in agreement with our in vitro data that suggested administration of vehicle may have protective properties on the pulmonary epithelial barrier. Exposure to vehicle also reduced the agonistic IL-6/sIL6r and increased the antagonistic sgp130 compared with the serum from naive rats. Third, analysis of serum collected from rats instilled with MWCNT revealed that sIL6r and IL-6 increased, whereas sgp130 significantly decreased below detectable levels. We suggest that levels of IL-6 transsignaling agents in serum from the MWCNT group would have increased the probability that IL-6 complexes with sIL-6r, resulting in activation of membrane gp130 and signal transduction.

Immunohistochemical analysis of rat aortas collected 24 h after instillation indicated that the MWCNT group had a noticeable staining for gp130 along the endothelial lining. Aortas collected from vehicle-instilled rats had staining similar to what we observed with our negative control. It has been documented that in vitro activation of membrane gp130 increases gp130 expression in smooth muscle (22) and endothelial cell cultures (45). Although gp130 staining differences in the aortic sections from vehicle and MWCNT exposed rats were most dramatic in the endothelial layer, staining was also present throughout the wall of the aorta, supporting reports of gp130 expression in smooth muscle (22).

The activation of NF-κB by IL-6 is established (11, 13) and we sought to examine cardiopulmonary tissues for a link between expression of gp130 and NF-κB/COX-2. Linking gp130 expression to changes in expression of NF-κB/COX-2 might elucidate the mechanistic link between IL-6 transsignaling and the reported cardiovascular detriments associated with pulmonary exposure to MWCNT. Cox-2 has been linked to airway remodeling in mice (47) and altered coronary artery vasocontraction in rats (59). The downregulation of lung gp130 did not correspond to changes in expression patterns of NF-κB and COX-2. We found no evidence of a change in gp130 mRNA and protein expression in the heart but we did find corresponding downregulation of Nfκb and COX-2 mRNA in the heart. In the aortas, only gp130 and Nfκb mRNA were upregulated. Although our data appear to conflict with the hypothesis that exposure to MWCNT would result in upregulation of NF-κB and COX-2, it is likely that examination of 1) nuclear fractions of NF-κB p65 and COX-2 activity and 2) cell-specific expression/activity of NF-κB and COX-2 would reconcile these findings. We speculate that downregulation of Nfκb and COX-2 mRNA in the heart may have been an attempt to dampen increased activity of these proteins; however, this was not evaluated in our study.

Of the tissues investigated, evidence of changes in protein expression associated with changes in mRNA expression were

Fig. 10. Hypothesized mechanism: IL-6 transsignaling mediation of cardiovascular dysfunction following pulmonary exposure to MWCNT. Inhalation of MWCNT results in increased lung permeability and release of IL-6 and sIL-6R into serum. MWCNT exposure also reduces the serum pool of sgp130 impairing endogenous antagonization of IL-6 transsignaling. Simultaneous upregulation of membrane bound gp130 in the heart and vasculature results in effector tissue IL-6 signal transduction, resulting in increased expression of NF-κB and COX-2 via JAK/STAT pathways.
the homogeneous aortic upregulation of gp130 mRNA and gp130 protein expression in aortic endothelial cells and 2) the homogeneous downregulation of gp130 mRNA and protein in lung tissue. The relationship of these findings to decreased serum sgp130 following MWCNT instillation raise a provoking question: Is the decrease in gp130 expression in the lung responsible for decreased serum sgp130? If the lung normally sheds sgp130 into the bloodstream and pulmonary insult results in decreased gp130 expression, the resulting decrease in sgp130 could be an important response mechanism that contributes to a cardiovascular susceptibility to injury following pulmonary exposures.

Lastly, alveolar epithelial cells and human fibroblasts respond to gp130 activation by increasing gp130 turnover, including increased gp130 protein degradation and synthesis (1). During these processes gp130 is highly modified by ubiquitination, truncation (1), and glycosylation, yielding Western blot banding from 90 to 130 kDa (67). In agreement we report gp130 antibody-positive bands from 90 to 130 kDa in our Westerns and we found decreased gp130 protein expression in the lung following MWCNT instillation. This coincided with a twofold downregulation in gp130 mRNA in homogenized lung tissue collected from MWCNT-instilled rats. Conversely, although lung homogenates showed downregulated gp130 mRNA, isolated epithelial cells demonstrated modest upregulation of gp130 mRNA, suggesting differing roles in cellular vs. tissue level responses to MWCNT.

In conclusion, we have presented evidence that pulmonary exposure to MWCNT can increase lung permeability, downregulate expression of gp130 in the lungs, decrease circulating sgp130 concentration, and upregulate aortic endothelial cell expression of gp130 through a potential linkage with IL-6 signaling (Fig. 10). These findings contribute to a novel understanding of how lung tissue responds to MWCNT. This study supports the rationale that pulmonary exposure to MWCNT can drive negative cardiovascular and systemic end points, possibly via IL-6 transsignaling. More work is required to complete an understanding of how pulmonary exposure to MWCNT contributes to cardiovascular sensitivity to inflammatory signals, through an IL-6 transsignaling mechanism. As the list of potential airborne toxichants that contribute adverse cardiovascular events grows, identifying a common mechanistic culprit would assist in toxicological screening and assessing exposure risk.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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