Monocyte adhesion induced by multi-walled carbon nanotubes and palmitic acid in endothelial cells and alveolar–endothelial co-cultures

Yi Cao¹, Martin Roursgaard¹, Nicklas Raun Jacobsen², Peter Møller¹, and Steffen Loft¹

¹Section of Environmental Health, Department of Public Health, University of Copenhagen, Copenhagen, Denmark and ²The National Research Centre for the Working Environment, Copenhagen, Denmark

Abstract
Free palmitic acid (PA) is a potential pro-atherogenic stimulus that may aggravate particle-mediated cardiovascular health effects. We hypothesized that the presence of PA can aggravate oxidative stress and endothelial activation induced by multi-walled carbon nanotube (MWCNT) exposure in vitro. We investigated the interaction between direct exposure to MWCNTs and PA on THP-1 monocyte adhesion to human umbilical vein endothelial cells (HUVECs), as well as on indirect exposure in an alveolar–endothelial co-culture model with A549 cells and THP-1-derived macrophages exposed in inserts and the effect measured in the lower chamber on HUVECs and THP-1 cells. The exposure to MWCNTs, including a short (NM400) and long (NM402) type of entangled fibers, was associated with elevated levels of reactive oxygen species as well as a decrease in the intracellular glutathione concentration in HUVEC and A549 monocultures. Both effects were found to be independent of the presence of PA. MWCNT exposure significantly increased THP-1 monocyte adhesion to HUVECs, and co-exposure to PA aggravated the NM400-mediated adhesion but decreased the NM402-mediated adhesion. For the co-cultures, the exposure of A549 cells did not promote THP-1 adhesion to HUVECs in the lower chamber. When THP-1 macrophages were present on the cell culture inserts, there was a modest increase in the adhesion and an increase in interleukin-6 and interleukin-8 levels in the lower chamber whereas no tumor necrosis factor was detected. Overall, this study showed that direct exposure of HUVECs to MWCNTs was associated with oxidative stress and monocyte adhesion and the presence of PA increased the adhesion when exposed to NM400.

Introduction
Exposure to air pollution particulate matter (PM) and engineered nanomaterials (ENMs) has been associated with vasomotor dysfunction and atherosclerotic plaque progression, possibly associated with particle-induced oxidative stress and inflammation (Donaldson et al., 2013; Møller et al., 2011). It has been shown that a diet with high content of carbohydrates or lipids exacerbated nanosized carbon black induced pulmonary and systemic inflammation in mice (Gotz et al., 2011). Oral exposure to nanosized carbon black aggravated the existing endothelial dysfunction in dyslipidemic Zucker rats (Folkman et al., 2012). Another study showed that obese mice had a higher level of systemic inflammation when compared with lean mice after exposure to diesel exhaust particles in the airways (Yanagisawa et al., 2014). Combined, these studies indicate that the metabolic status influences the health variables after exposure to particles.

The immune system can be activated by excessive nutrients in chronic metabolic diseases, thus linking nutrient metabolism to chronic inflammation (Gregor & Hotamisligil, 2011; Hotamisligil & Erbay, 2008). Indeed, elevated circulating free fatty acid (FFA) concentrations have been implicated in the development of metabolic diseases and atherosclerosis (Boden, 2008). Infusion of lipids in human blood increased the concentration of cell adhesion molecules, indicating activation of endothelial cells (Mathew et al., 2010). This is supported by in vitro studies showing that exposure to FFA promoted monocyte adhesion to endothelial cells (Zhang et al., 2006). Furthermore, exposure to high concentrations of palmitic acid (PA), an important circulating saturated fatty acid, can induce oxidative stress and an inflammatory response in human microvascular endothelial cells (Cheng et al., 2012; Maloney et al., 2009).

Multi-walled carbon nanotubes (MWCNTs) are high aspect ratio ENMs, characterized by a high length to width ratio, with unique features in regard to deposition, clearance, and pulmonary toxicity. Inhaled fibers, such as asbestos fibers, have been shown to migrate to the pleura, and promote the development of mesothelioma especially through macrophage-mediated inflammatory response, and MWCNTs are speculated to be able to do the same (Donaldson et al., 2010; Murphy et al., 2012). Oxidative stress is considered to be an important mechanism of action for carbon nanotube-mediated health effects (Shvedova et al., 2012). Airway exposure to MWCNTs in animals have shown impairment of endothelium-dependent vasodilation in coronary arteries (Stapleton et al., 2012), accelerated plaque progression in aorta (Cao et al., 2014), and exacerbated myocardial infarction after ischemia/reperfusion injury (Thompson et al., 2014; Urankar et al., 2012). In vitro model systems have
shown that MWCNT exposure can induce the production of reactive oxygen species (ROS) and the expression of adhesion molecules in cultured endothelial cells (Cao et al., 2014; Pacurari et al., 2011; Vidanapathirana et al., 2012). This suggests that a direct interaction between MWCNTs and endothelial cells is hazardous, although there is little translocation of nanomaterials from the lungs to the vascular system. The crosstalk between airway exposure to particles and vasomotor dysfunction is unresolved, although it may involve signaling by cytokines or other bioactive factors from the lung tissue. For instance, it has been shown that serum from ozone-exposed mice could impair the vasodilation response in aorta rings ex vivo (Robertson et al., 2013). This exposure situation can be mimicked in co-culture systems where a lung epithelial barrier consisting of lung epithelial cells separates particles from vascular cells (Rothen-Rutishauser et al., 2007). One such set-up is the Transwell co-culture system where cells in the lower chamber (e.g., representing the circulation) are separated from cells in the upper chamber (e.g., representing the alveolar space) by a porous membrane on an insert in the cell culture well.

We hypothesized that the presence of PA can aggravate oxidative stress and endothelial activation induced by MWCNT exposure in vitro. In this study, we examined the effects of MWCNT exposure on oxidative stress and monocyte adhesion to human umbilical vein endothelial cells (HUVECs), with emphasis on the interaction with PA, whereas oxidative stress measured as intracellular ROS and glutathione (GSH) level was determined in HUVECs and A549 monocultures. In addition, an alveolar–endothelial Transwell co-culture model with two compartments separated by a porous membrane was used to assess the monocyte adhesion to HUVECs in the lower chamber with the presence of PA, and induced by MWCNT exposure to A549 epithelial cells and THP-1-derived macrophages in the upper compartment. The protein level of tumor necrosis factor (TNF), interleukin (IL)-6 and IL-8 was measured in the medium from the lower chamber to indicate inflammatory responses. The adhesion of THP-1 monocytes to HUVECs was used as a major endpoint since the recruitment of monocytes is an important characteristic in the pathogenesis of atherosclerosis (Moore & Tabas, 2011), and increased monocyte adhesion to HUVECs has been observed by in vitro exposure to, e.g., wood smoke particles (Forchhammer et al., 2012) as well as MWCNTs (Cao et al., 2014).

Methods

Cell lines

The HUVECs were purchased from Invitrogen (Carlsbad, CA) and were cultured in Endothelial Cell Growth Medium Kit with 2% serum (Cell Applications, San Diego, CA). The monocytic cell line THP-1 and type II pneumocytes (A549) were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI or F-12 medium with 10% serum as previously described (Danielsen et al., 2009; Jantzen et al., 2012). The THP-1 cells were differentiated into adherent macrophage-like cells (denoted THP-1a) by treatment with 10ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) overnight. The THP-1a cells were attached to the surface of the culture flasks, whereas THP-1 cells stay in suspension and were removed with the supernatant. It has been shown that PMA-differentiated THP-1 macrophages resemble primary human monocyte-derived macrophages (Daigneault et al., 2010).

Particles

MWCNTs used in the present study were from two different suppliers, coded as NM400 (from Nanocyl, Auvelais, Belgium) and NM402 (from Arkema Graphistrength, France) in the European Commission Joint Research Centre Nanomaterials Repository (http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository). The characterization of MWCNTs has been reported elsewhere (Kermanizadeh et al., 2013; Vietti et al., 2013) and data are summarized in Table 1. MWCNT stock solution was prepared by sonication a 2.56 mg/ml suspension of particles in double-distilled water (Sigma-Aldrich, St. Louis, MO) containing 2% bovine serum (Sigma-Aldrich, St. Louis, MO), using a Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT) equipped with a disrupter horn (model number: 101-147-037) before each experiment. The suspension was sonicated continuously for 16 min at amplitude of 10% and cooling on ice to avoid sample heating and evaporation. After sonication, the suspension was diluted and used only once. The structure of MWCNTs in suspension before and after sonication was investigated by scanning electron microscopy as recently described (Cao et al., 2014).

To determine an approximate size of the particles in suspension, analysis was performed with a NanoSight LM20 (NanoSight, Amesbury, UK) in triplicates on three independent days. About 4 μg/ml particles were suspended in distilled water, 0.2% bovine serum albumin (BSA), or 100 μM PA + 0.2% BSA to determine the effects of different kinds of solvents (see below). The Nanoparticle Tracking Analysis (NTA) version 2.0 (Thermo Scientific, Fremont, CA) was used to track and analyze the particles and the sizes of particles are an approximation to their sphere-equivalent hydrodynamic diameter describing the movement of a corresponding spherical particle in the solvent.

Preparation of palmitic acid

The PA stock solution (200 mM) was prepared in 96% ethanol (EtOH) at 37°C and stored at –20°C. On the day of use, 5 mM PA suspension was made in Hanks solution containing 10% BSA at 37°C for about 60 min including vortexing for 5–10 min, and then further diluted in HUVEC medium to a final concentration of 100μM for exposure. The molar ratio of PA to BSA is approximately 3:3:1. The final concentrations of EtOH and BSA in exposure medium were 0.05% and 0.2%, respectively. An equal volume of the Hanks solution/EtOH/10% fatty acid-free BSA was used as control.

Table 1. Physico-chemical characterization of MWCNTs.

| Characteristic     | NM400       | NM402       |
|-------------------|-------------|-------------|
| BET surface area (m²/g) | 298         | 225         |
| Length (nm)      | 700–3000   | 400–4000    |
| Diameter (nm)    | 5–35       | 6–20        |
| Impurities (%)   | 5.4%       | 3.2%        |
| Aluminum (%)     | 1.0%       | 3%          |
| Iron (%)         | 0.2%       | 1.6%        |
| NTA size Mean (nm) | 222 ± 38 (water) | 271 ± 29 (water) |
|                  | 246 ± 41 (BSA) | 312 ± 25 (BSA) |
|                  | 242 ± 38 (PA)  | 282 ± 12 (PA)  |
| Mode (nm)        | 87 ± 6 (water) | 177 ± 46 (water) |
|                  | 108 ± 17 (BSA) | 255 ± 18 (BSA) |
|                  | 121 ± 31 (PA)  | 252 ± 33 (PA)  |

*From Kermanizadeh et al. (2013).*
*From Vietti et al. (2013).*
*Characterized by the joint action Nanogenotox project (http://www.nanogenotox.eu/).*
*The size of MWCNTs in suspension measured by nanoparticle tracking analysis (NTA) in the present study. The “Mean” reflects the mean size of the particles, and the “Mode” reflects the size of the most frequently occurring particles. Data were expressed as mean ± SEM of three independent experiments (n = 3 for each experiment).*
Alveolar–endothelial co-culture model

The experimental setup of the co-culture model is illustrated in Supplemental Figure S1. Fifty thousand A549 cells were seeded per well on 0.1% gelatin pre-coated BD Falcon cell culture inserts (surface area: 0.9 cm², diameter of pores: 0.4 µm, transparent PET membrane for 12-well plate format; BD Biosciences, Erembodegem, Belgium). The cells were cultured for 7 d with 1 ml medium in the upper and 2 ml in the lower chamber, and medium was changed every 2nd or 3rd day. On day 6, 2 × 10⁵ HUVECs were seeded per well in a separate 12-well plate and cultured overnight. On day 7, the old medium was removed, and HUVECs in 12-well plate were incubated with 100 µM PA, 2 × 10⁵ CellTracker™ Green labeled THP-1 cells per well (see below) and then co-cultured with the cell culture inserts containing the A549 cells. One ml of MWCNTs (8 µg/ml) or TNF (100 ng/ml, positive control) was added into the upper chamber. Because we observed no changes of adhesion by exposure of both types of MWCNTs to inserts with only A549 monolayer, THP-1a cells were also co-cultured with A549 monolayer to promote adhesion. On day 6, 1 × 10⁵/TNP-1a cells were co-cultured with A549 monolayer and the exposure was done as indicated above. For comparison, cell culture inserts with only 1 × 10⁵ TNT-1a cells were also included. After long-term cultivation, there are approximately 7 × 10⁵ A549 cells on the cell culture inserts. The A549:TNP-1a ratio in this study is about 7:1, which is close to normal human pneumocytes:macrophages ratio (9:1) (Jantzen et al., 2012).

The design in this study tried to use an intelligent approach to investigate many parameters in a simple one culture setup with dose–response effects here, and then select a lower number of relevant responses tested in the co-culture. This approach was used to optimize the use of the co-culture in case of relevance and to select relevant dosing.

Transepihelial electrical resistance (TEER) measurement

TEER was measured with an Endohm 12 chamber and an EVOM voltohmmeter (World Precision Instruments Ltd, Stevenage, UK) containing 2 ml A549 medium to indicate the A549 epithelial integrity. The TEER of inserts without cells was subtracted from all samples, and the values were expressed as Ω·cm². For the quality control of the co-culture, TEER was measured over 8 d in the insert and results are shown in Supplemental Figure S2. To determine the changes of TEER of the samples before and after MWCNTs or TNF treatment, TEER was measured on day 7 before co-culturing with HUVECs and TNP-1 cells and on day 8 after the exposure.

FITC-dextran permeability assay

To assess the permeability of FITC-dextran (Mw 40,000) across the inserts and A549 monolayers, 1 ml 10 µg/ml FITC-dextran (Sigma-Aldrich, St. Louis, MO) in A549 medium was added into the upper chamber of inserts with or without A549 cells on day 7. On day 8, 100 µl of medium both from upper and lower chambers were transferred to a black 96-well plate, and measured in triplicates for each sample. The fluorescence was measured at ex 485 nm and em 538 nm in a fluorescence spectrophotometer (Fluoroskan Ascent FL; Labsystems, Vienna, VA) and the percentage of FITC-dextran permeability was calculated. Results are shown in Supplemental Figure S3.

Cytotoxicity assay

The cytotoxicity of HUVECs and A549 monocultures was measured using the WST-1 assay according to instructions of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany). This method measures mitochondrial dehydrogenase activity in living cells. For HUVECs, 2 × 10⁴ cells in 0.1% gelatin pre-coated 96-well plates were exposed to various concentrations of MWCNTs with or without the presence of 100 µM PA for 24 h. After exposure, the cells were rinsed once with Hanks solution, incubated with 100 µl fresh medium containing 10% WST-1 reagent for 2 h, and the absorbance was then measured at 450 nm with 630 nm as a reference by an ELISA reader (Labsystems, Vienna, VA, Multiskan Ascent) (Vesterdal et al., 2012). For A549 cells, 5 × 10⁴ cells in 96-well plates were exposed to MWCNTs for 24 h before WST-1 assay.

The cytotoxicity of MWCNTs in the alveolar–endothelial co-culture was assessed by transferring the inserts to new 12-well plates containing 2 ml fresh A549 medium. The inserts were rinsed once with 1 ml Hanks solution and then incubated with 1 ml A549 medium containing 1% WST-1 reagent in the upper chamber for 2 h. After incubation for 2 h, medium containing product was transferred to a 96-well plate, and the absorbance was measured in triplicates at 450 nm with 630 nm as a reference by an ELISA reader (Labsystems, Vienna, VA).

Cell proliferation assay

The proliferation of HUVEC monoculture was measured with the BrdU cell proliferation assay kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions of the manufacturer. Briefly, 5 × 10⁵ HUVECs were exposed to MWCNTs with or without PA in the presence of BrDU for 24 h and the incorporation of BrdU into DNA of proliferating cells was measured according to the instructions.

ROS measurement

The total ROS production of HUVECs in monoculture was measured both quantitatively and semi-quantitatively using the ROS sensitive probe CM-H₂DCFDA, which is oxidized into the fluorescent dye DCF upon reaction with ROS. In our previous work, we have incubated the probe-loaded cells with the particles and continuously measured the ROS for 3 h (Cao et al., 2014; Forchhammer et al., 2012). However, preliminary experiments showed that the presence of BSA could interfere with the assay. Therefore, we measured the ROS levels after 24 h exposure to MWCNT. For the quantitative ROS measurement, 2 × 10⁴ HUVECs in 0.1% gelatin pre-coated 96-well black plates were exposed to various concentrations of MWCNT with or without PA for 24 h. After exposure, the cells were rinsed once with Hanks solution and ROS measurement was done as previously described (Cao et al., 2014; Forchhammer et al., 2012; Vesterdal et al., 2012). Briefly, the cells were stained with 2 µM CM-H₂DCFDA in Hanks solution for 15 min, rinsed twice, and the fluorescence was then measured every 15 min for 3 h at ex 485 nm and em 538 nm in a fluorescence spectrophotometer.

For the quantitative ROS measurement in A549 monoculture, 5 × 10⁴ cells in 96-well black plates were exposed to MWCNTs for 24 h, rinsed, and then total ROS was determined as described above.

For the semi-quantitative ROS measurement, 2 × 10⁴ HUVECs were grown on 0.1% gelatin pre-coated 8-well microscopy chamber slides (Ibidi, Munich, Germany) and exposed to 8 µg/ml MWCNT with or without PA for 24 h. After exposure, the cells were rinsed media, stained in 25 µM DCFH and Hoechst 33342 in serum-free media for 30 min, rinsed twice with media, and then examined by combined differential interference contrast (DIC) and fluorescence microscopy in a Leica AF6000 inverted wide-field microscope with a 40 times dry objective with NA 0.7 (Leica Microsystems GmbH, Wetzlar, Germany). For each
experimental condition, images were obtained in 10 randomly selected independent fields. Representative ROS fluorescence pictures are shown in Supplemental Figure S7. The DCF-integrated density was measured using ImageJ (NIH, Bethesda, MD) and data were normalized to cell number. By this way approximately 300 cells were counted.

**Intracellular-reduced GSH**

The intracellular-reduced GSH concentration was measured by using ThioGlo-1 (Covalent Technologies, Inc., Walnut Creek, CA), which is fluorescent upon the reaction with intracellular low molecular weight thiols (Cao et al., 2011). Because most of the low molecular weight thiols are reduced GSH, ThioGlo-1 could be used to measure intracellular reduced GSH. Briefly, 2 x 10^4 HUVECs or 5 x 10^4 A549 cells after 24 h exposure were rinsed once in PBS, incubated with ThioGlo-1 for approximately 5 min and then measured by fluorescence spectrometer at exc 355 nm and em 460 nm. By this short-term incubation, ThioGlo-1 is not accessible to high molecular weight thiols such as protein thiols. The data were compared with a GSH standard curve and expressed as nmol GSH per 10^6 cells.

**Cell adhesion assay**

The in vitro cell adhesion assay of THP-1 monocytes to HUVECs was done by using CellTracker™ Green CMFDA (5-Chloromethylfluorescein Diacetate, Invitrogen, Carlsbad, CA). The THP-1 cells were labeled with 10 μM CellTracker™ Green for 30 min in serum-free medium according to instructions of the manufacturer for cell staining. 2 x 10^4 HUVECs on 0.1% gelatin pre-coated 96-well black plates were exposed to various concentrations of MWCNT with or without 100 μM PA for 24 h. After exposure, the HUVECs were washed once with Hanks solution and labeled THP-1 cells (5 x 10^3/well cells) were added to HUVECs and incubated for 1 h. Unbound THP-1 cells were subsequently washed away in Hanks solution and the fluorescence of bound THP-1 was measured at ex 485 nm and-em 538 nm in a fluorescence spectrophotometer.

To investigate whether oxidative stress or IL-6 signaling were involved in the adhesion effects, we performed an additional adhesion assay with attachment of monocytes to HUVECs after exposure to MWCNTs in combination with 1 mM 50 PA for 24 h. After exposure, the cells were rinsed twice in Hanks solution and labeled THP-1 cells (5 x 10^4/well cells) were added to HUVECs and incubated for 1 h. Unbound THP-1 cells were washed away in Hanks solution and the fluorescence of bound THP-1 was measured at ex 485 nm and-em 538 nm in a fluorescence spectrophotometer. For each experimental condition, images were obtained in 10 randomly selected independent fields. Representative images are shown in Supplemental Figure S8. The number of labeled THP-1 cells in each field was counted by using ImageJ (NIH, Bethesda, MD) and data were normalized as cell number/field.

For the quantitative adhesion assay in the co-culture, the inserts were removed after exposure, and the HUVECs and THP-1 cells in the lower chamber were rinsed twice in Hanks solution to remove the non-bound THP-1 cells. After that, the fluorescence of the whole plate was scanned in a fluorescence spectrophotometer, and a mean value of the fluorescence in each well was calculated to indicate the bound THP-1 cells.

**Cytokine measurements**

After exposure, the medium from the lower chamber of co-cultures was centrifuged and the supernatants were stored at -80 °C before analysis. Protein levels of TNF, IL-6, and IL-8 in 50 μl of supernatants were measured using a multiplex Cytometric Bead Array Flex system acquired with a FACS Array (BD Biosciences, Emmelgem, Belgium) and FCAP Array software according to the instruction of the manufacturer. The detection limits of the different proteins are 1.6 pg/ml for IL-6 and 1.2 pg/ml for TNF and IL-8. The results are reported as concentration (pg/ml), whereas we have used the fold-difference between exposed and controls for the statistical analysis.

**Statistics**

The results were analyzed by full-factor ANOVA followed by the post-hoc least statistical difference analysis to evaluate statistically significant interactions between PA and MWCNT treatment. The results on THP-1 cell adhesion to HUVECs in Transwell co-culture system were analyzed by full-factor ANOVA with exposure and cell type as categorical variables. The results of TEER measurements were analyzed by repeated measurements ANOVA. The results on cytokine secretion was analyzed by nested ANOVA with the type of MWCNT (NM400 or NM402) nested in the experimental system. The cytokine responses were analyzed as fold-differences because there was a relatively high inter-day variation between the basal production of IL-6 and IL-8. p Values <0.05 were considered to be statistically significant. The statistical analysis was done in Statistica 5.5 from StatSoft, Inc., Tulsa, OK. All data are presented as means and standard error of the mean (SEM) values.

**Results**

**Characterization of MWCNTs**

Figure 1 shows scanning electron microscopy images of NM400 and NM402 in dispersion vehicle before and after sonication. Both types of MWCNTs consisted of entangled, irregular and bend nanotubes. Table 1 summarizes the physico-chemical characterization of MWCNTs from this study as well as published references (Kermanizadeh et al., 2013; Vietti et al., 2013). The Nanoparticle Tracking Analysis (NTA) indicated that NM400 suspensions were in general recognized as smaller particles than the NM402 suspensions as indicated both by the mean size (222 ± 38 nm versus 271 ± 29 nm) and by the mode (87 ± 6 nm versus 177 ± 46 nm). The presence of BSA or PA showed an increase in the mean and the mode size of the MWCNTs, which was more pronounced for NM402 than NM400.

**Cytotoxicity and proliferation ability**

There was very low cytotoxicity when cells were exposed to both NM400 and NM402, the detailed statistical analysis is shown and described in Supplemental Figures S5 (WST-1) and S6 (proliferation).

**Intracellular ROS levels**

The increase in intracellular ROS levels induced by MWCNT exposure is shown in Figure 2. For HUVECs monoculture, there were interactions between the concentration and the type of MWCNT (p < 0.001), whereas the co-treatment with PA had no effect (p > 0.05). The exposure to NM400 was associated with substantially increased ROS at all concentrations (p < 0.05), whereas the NM402 only increased the ROS levels at 32 μg/ml.
Figure 1. Scanning electron microscopy images of NM400 (A and B) and NM402 (C and D) before (A and C) or after sonication (B and D).

Figure 2. Intracellular ROS levels after 3 h exposure to MWCNTs. (A) ROS levels in HUVECs after MWCNT exposure in medium with or without palmitic acid (PA). (B) The fold increase of ROS (DCFH fluorescence/cell) in HUVECs assessed by fluorescence microscopy from three independent experiments with 10 independent fields for each experiment. (C) The ROS levels in A549 monoculture after 3 h exposure to MWCNTs in medium with or without PA. Bars are means ± SEM of 4–5 independent experiments. *p < 0.05 compared with the unexposed cells.
The ROS level was further assessed by fluorescence microscopy of HUVECs exposed to 8 μg/ml MWCNT with or without PA in the cell medium (representative images are shown in Suplemental Figure S7). The results showed that only NM400 increased the ROS levels in the presence of PA $(p<0.05)$, whereas NM402 had no effect on the levels of ROS (Figure 2B).

For A549 monocultures, there were interactions between the concentration and the type of MWCNT $(p<0.01)$. The exposure to NM400 was associated with increased ROS levels at all concentrations $(p<0.05)$, whereas the NM402 did not significantly affect the ROS levels (Figure 2C).

Intracellular-reduced GSH

Figure 3 shows the effect of MWCNT exposure on the intracellular GSH concentrations. For HUVECs, there was a concentration-dependent decrease in GSH concentration $(p<0.01)$, but no effect of the presence of PA. The GSH level was significantly reduced in HUVECs exposed to NM400 (8–32 and 16–32 μg/ml with or without PA, respectively, $p<0.05$). There was significantly reduced intracellular GSH concentration at all concentrations of NM402 $(p<0.05)$, which was independent of the presence of PA (Figure 3A).

For A549 cells, the exposure to both NM400 and NM402 significantly decreased the GSH concentration at all concentrations $(p<0.01)$ (Figure 3B).

Adhesion assay

Figure 4(A) shows the THP-1 adhesion to HUVECs after 24 h direct exposure to MWCNTs. The adhesion followed a

| Concentration (μg/ml) | Control | NM400-PA | NM400+PA | NM402-PA | NM402+PA |
|-----------------------|---------|-----------|----------|----------|----------|
| 0                     | 100     | 150       | 200      | 250      | 300      |
| 2                     | 100     | 150       | 200      | 250      | 300      |
| 4                     | 100     | 150       | 200      | 250      | 300      |
| 8                     | 100     | 150       | 200      | 250      | 300      |
| 16                    | 100     | 150       | 200      | 250      | 300      |
| 32                    | 100     | 150       | 200      | 250      | 300      |
| Control               | 100     | 150       | 200      | 250      | 300      |

* $p<0.05$ compared with the unexposed cells.

| Concentration (μg/ml) | Control | NM400 | NM402 |
|-----------------------|---------|-------|-------|
| 0                     | 100     | 150   | 200   |
| 2                     | 100     | 150   | 200   |
| 4                     | 100     | 150   | 200   |
| 8                     | 100     | 150   | 200   |
| 16                    | 100     | 150   | 200   |
| 32                    | 100     | 150   | 200   |

* $p<0.05$ compared with the unexposed cells.

| Concentration (μg/ml) | Control | NM400 | NM402 |
|-----------------------|---------|-------|-------|
| 0                     | 100     | 150   | 200   |
| 2                     | 100     | 150   | 200   |
| 4                     | 100     | 150   | 200   |
| 8                     | 100     | 150   | 200   |
| 16                    | 100     | 150   | 200   |
| 32                    | 100     | 150   | 200   |

* $p<0.05$ compared with the unexposed cells.

Figure 4. Adhesion of THP-1 cells to HUVECs after 24 h exposure to MWCNTs. (A) Percentage of THP-1 cell adhesion to HUVECs exposed to MWCNTs in medium with or without palmitic acid (PA). (B) Number of adherent THP-1 cells/fields after direct exposure to 8 μg/ml MWCNTs assessed by fluorescence microscopy from three independent experiments with six independent fields for each experiment. (C) Adhesion of THP-1 cells to HUVECs in the Transwell co-culture model after 24 h exposure to 8 μg/ml MWCNTs. Bars are means ± SEM of 3–4 independent experiments.

* $p<0.05$ compared with the unexposed cells. 

*(Y. Cao et al. Nanotoxicology, 2016; 10(2): 235–244)*
bell-shaped concentration–response curve with increased adhesion at the middle concentrations, whereas the high concentrations (16 and 32 μg/ml) showed lower adhesion. For the direct exposure, there was a triple interaction among the concentration, type of MWCNT, and co-treatment with PA ($p < 0.001$). This showed that NM400 exposure increased the adhesion without the presence of PA (2–16 μg/ml, $p < 0.05$). The co-treatment with PA was associated with a further increase in the adhesion as compared with cells that were only exposed to NM400 (4–8 μg/ml, $p < 0.05$). The direct exposure to NM402 only increased the adhesion of THP-1 cells to HUVECs (2–8 μg/ml, $p < 0.05$), whereas co-treatment with PA abrogated the NM402-induced cell adhesion at 4 and 8 μg/ml ($p < 0.05$). NM400 and NM402 generated the same level of adhesion in medium without PA, whereas NM400 was more potent than NM402 in medium with PA (2–8 μg/ml, $p < 0.05$) (Figure 4A).

The addition of the antioxidant NAC or IL-6 as a co-factor during exposure to the MWCNTs did not significantly change the adhesion of THP-1 cells to the HUVEC cells (Supplemental Figure S9).

The adhesion was further assessed by microscopy (representative images are shown in Supplemental Figure S8). The results showed that the presence of PA increased the adhesion of THP-1 cells to HUVECs ($p < 0.05$). The exposure to NM400 and NM402 also increased the adhesion ($p < 0.05$), whereas there was no interaction between PA and particle treatment in the full-factorial ANOVA (Figure 4B).

Figure 4(C) shows the results from experiments of the Transwell co-culture system where the MWCNTs were applied to the inserts with or without A549 and/or THP-1a cells. The lower chamber contained HUVECs and THP-1 cells in medium with PA. The presence of A549 monolayer in the upper chamber did not affect the adhesion of THP-1 cells to HUVECs in the lower chamber, whereas there was an interaction between the presence of THP-1a cells and the exposure to MWCNTs ($p < 0.05$). The presence of THP-1a cells increased the NM400- and NM402-mediated adhesion of THP-1 cells to HUVECs in the lower chamber ($p < 0.05$). The addition of 100 ng/ml of TNF to the inserts with A549 monolayer was associated with a 70% increased THP-1 adhesion to HUVECs ($p < 0.05$; results not shown).

**Release of cytokines**

The concentration of secreted IL-6 and IL-8 was higher in the lower chamber of the Transwell co-culture system when THP-1a cells were present in the upper chamber (Figure 5). The statistical analysis indicated that the IL-8 response depended on the type of cell culture on the inserts, with strongest responses with THP-1a cells present ($p < 0.05$, nested ANOVA). The exposure to NM400 with THP-1a cells present was associated with a 33% increase in IL-8 content in the lower chamber (41% (95% confidence interval: 16–66%) higher IL-8 when based on the fold difference in the nested ANOVA) (Figure 5B). There was a similar response on the level of IL-6 as for the level of IL-8, although this was only of borderline statistical significance in the overall nested ANOVA ($p = 0.06$). Nevertheless, the post hoc test indicated that exposure to NM400 resulted in 11% (95% CI: 1–21%) higher IL-6 level in the lower chamber of the experimental system when only THP-1a cells were on the insert. The TNF concentrations in medium from the lower chamber were all below the detection limit (1.2 pg/ml).

**Epithelial barrier function**

The transepithelial electrical resistance (TEER) development was followed during 8 d of culturing of A549 cells in the Transwell co-culture system to assess the barrier function. It reached a plateau at day 7 of the culture (Supplemental Figure S2). This was further evidenced by assessment of the permeability of FITC-dextran after 8 d of culturing where the percentage of translocation of FITC-dextran was 2.4 ± 0.18% and 0.6 ± 0.05% for inserts without and with A549 monolayer, respectively ($p < 0.001$, Supplemental Figure S3).

The TEER value of the A549 monolayer on the inserts increased during the co-culturing with HUVECs and THP-1 cells ($p < 0.001$), whereas the exposure to MWCNTs or TNF had no significant effect on the TEER value ($p = 0.58$) (Figure 6).

**Discussion**

In the present study, we showed that direct exposure to MWCNTs is associated with increased adhesion of THP-1 monocytes to HUVECs in co-culture, whereas this effect was not observed in co-cultures where the MWCNTs were separated from THP-1 and endothelial cells by cell culture inserts with or without an A549 epithelial cell barrier unless THP-1-derived macrophages were present above the barrier. There was increased adhesion of THP-1 cells onto HUVECs in co-cultures that were co-treated with NM400 and PA, whereas PA decreased NM402-mediated THP-1 cell adhesion to HUVECs. The concentration of PA (100 μM) was chosen because it is similar to the level in human blood (Yi-Jama et al., 2002), whereas higher concentrations may cause apoptosis of HUVECs (Kim et al., 2010). The WST-1 and BrdU proliferation assay showed that there was little cytotoxicity in HUVECs that were exposed to 100 μM of PA. Exposure to 100 μM PA has been shown to induce inflammatory response, adhesion molecule expression, and ROS generation in human microvascular endothelial cells (Cheng et al., 2012; Maloney et al., 2009), but not in HUVECs (Chen et al., 2003; Holthe et al., 2005), which is
consistent with our results. The increase in adhesion could be due to the activation of kinase signaling pathways, which has been argued to be independent of adhesion molecule expression (Ewart et al., 2008; Huber et al., 2003). For instance, oxidized phospholipids could promote monocytes adhesion to HUVECs, which is dependent on protein kinases A and C and independent of adhesion molecules ICAM-1, VCAM-1, and E-selectin (Huber et al., 2006).

It has been shown that MWCNT exposure promoted adhesion molecule expression in human endothelial cells (Cao et al., 2014; Pacurari et al., 2011; Vidanapathirana et al., 2012). This is consistent with the observation of increased THP-1 adhesion to HUVECs by direct exposure of MWCNTs in the present study. The presence of PA further increased the adhesion induced by NM400, but not NM402, which indicated that the interaction between MWCNTs and PA depends on the type of MWCNTs. In the co-culture combining A549 epithelial cells and HUVECs, the exposure of A549 monolayer to either type of MWCNTs did not promote the adhesion of THP-1 cells to HUVECs in the lower chamber. The Transwell insert had a pore size of 0.4 μm, which impedes the transport of MWCNTs to the lower chamber (Klein et al., 2013). The permeability assay also showed low level of transport of FITC-dextran through the inserts with or without A549 monolayer during the 24-h exposure period. It indicates that the translocation of MWCNTs from the upper chamber to the lower chamber was negligible. Therefore, it is expected that MWCNT-mediated monocyte adhesion in the co-culture model is induced by the mediators secreted from the activated cells, rather than the translocation of MWCNTs from upper chamber to lower chamber.

The results from the Transwell co-culture model showed that MWCNTs did not activate A549 cells to secrete cytokines. THP-1a cells had both higher basal level of IL-6 and IL-8 secretion and the exposure to NM400 augmented especially IL-8 content in the lower chamber. As the human airways consist of many different kinds of immune cells, it is possible that the crosstalk between different types of immune cells may amplify the inflammatory response upon MWCNT exposure. For instance, there was significantly increased TNF and IL-8 release in both upper and lower chambers of a triple co-culture model consisting of 16HBE14o-epithelial cells, macrophages, and dendritic cells in response to 5–20 μg/ml MWCNT exposure (Clift et al., 2014). A model consisting of macrophages and A549 cells in the upper chamber and dendritic cells on the basal side of the membrane in the lower chamber showed substantial inflammatory signaling induced by MWCNT exposure (Gasser et al., 2012). It has also been shown that 1.2 μg/ml MWCNT exposure to small airway epithelial cells resulted in elevated expression of genes related to inflammation and release of inflammatory cytokines (Snyder-Talkington et al., 2013b) and also on activated human microvascular endothelial cells in the lower chamber of a co-culture system (Snyder-Talkington et al., 2013a). Therefore, small airway epithelial cells could be more capable of releasing inflammatory mediators than A549 cells, although the latter do respond with increased IL-6 and IL8 transcription at very high exposure concentrations of, e.g., diesel exhaust particles (Dybdahl et al., 2004). The exposure of lung epithelial cells to silica nanoparticles (NPs) in co-cultures induced inflammatory responses in endothelial cells on the opposite side of the insert as increased sICAM-1, IL-6, and IL-8 release, but most of the responses were absent at low concentrations (Kasper et al., 2011). Similarly, it was shown that exposure of A549 and THP-1 cells to silica NPs induced inflammatory responses in endothelial cells on inserts assessed as increased release of IL-6, IL-8, and MIP-1α, but not TNF (Napierska et al., 2012). This is in agreement with the lack of TNF secretion in the Transwell co-culture in our study.

The exposure to MWCNTs for 24 h in our study was associated with reduced GSH content in HUVECs and A549 cells. The reduced intracellular GSH content may occur as a consequence of increased ROS level induced by the MWCNT exposure. In keeping with this notion, we have previously observed that a 3 h incubation period with NM400 and NM402 was associated with a similar increase in intracellular ROS level (Cao et al., 2014). The intracellular ROS level in the present study was measured in HUVECs and A549 cells after a 24 h exposure period. As the MWCNTs were removed from the cells by changing the medium, it is mainly secondary intracellular sources of ROS that is measured in the post-exposure period. The ROS level in the present experiments, therefore, represents the ability of the cells to generate ROS, although direct ROS production by engulfed MWCNTs may also contribute to the overall level of ROS. Interestingly, the higher cellular ROS level in the NM400 exposed A549 (Figure 2C) and HUVECs (Figure 2A) coincides with this sample’s higher ability to promote cell adhesion in the presence of PA (Figure 4A and B). This is in keeping with other observations of reduced GSH levels in both cell monocultures and triple co-cultures by MWCNT exposure (Clift et al., 2014). The higher ROS level and cytokine secretion in the NM400 exposed cells suggest that oxidative stress and inflammation are implicated in the MWCNT-mediated interaction between THP-1 cells and HUVECs. Conflicting with this was the result that addition of either NAC or IL-6 during the exposure to MWCNTs was unable to significantly change the adhesion of THP-1 cells to HUVECs, indicating that additional factors are involved in the induction of adhesion.

The lung epithelial cells function as a barrier that prevents the translocation of inhaled particles into the circulation. In this study, we measured the TEER as a marker of the epithelial integrity, and the result showed that exposure to MWCNTs or TNF did not significantly affect the TEER, and had no obvious effects on cytoxicity of A549 monolayer on the inserts. Previous work has shown that NP exposure can decrease TEER, but only at relatively high concentrations. Lehmann et al. (2009) showed that diesel exhaust particles could modulate the tight junction protein occludin and decrease TEER of A549 monolayer on cell culture inserts, but only at high concentrations (125 μg/ml). Kasper et al. (2011) also showed that silica NPs significantly decreased TEER at high concentrations (600–6000 μg/ml), but not at low concentrations (6–60 μg/ml). In addition, our data showed an increase in TEER after co-culturing the A549 cells with HUVECs and THP-1 cells, which could be due to a synergistic effect of the interactions of different cell types, which might be mediated indirectly by mediators or by direct contact of monocytes adhering to the bottom of the inserts with possible transformation to macrophages.
It has been estimated that a full working lifetime exposure to 1 mg/m³ of carbon nanotubes with a length-to-diameter aspect ratio of 167 would correspond to exposure concentrations ranging from 12.4 to 46.5 μg/cm² lung surface (Gangwal et al., 2011). The concentration of MWCNT in the upper chamber in our setup (8 μg/ml) corresponds to 7.2 μg/cm² indicating that the concentrations used are within an obtainable exposure scenario in real life. The level of exposure directly to the endothelium is debatable since the possible translocation of MWCNTs to the blood stream is expected to be very low if any. A possible direct exposure would although be realistic in the case of MWCNTs used for drug delivery systems within nanomedicine.

Conclusions

In conclusion, the present study showed that direct exposure of MWCNTs to HUVECs could promote oxidative stress and THP-1 monocyte adhesion, and that there is an interaction between PA and MWCNT exposure on the adhesion, but not on oxidative stress. However, indirect exposure of MWCNTs to inserts with A549 monolayer in the upper chamber did not promote THP-1 adhesion in the lower chamber, whereas exposure of MWCNTs to inserts with THP-1-derived macrophages with or without A549 slightly increased THP-1 adhesion, indicating that the translocation of MWCNTs into the lower chamber is required to promote the adhesion unless immune cells such as macrophages present in the airways produce mediators upon exposure for secondary transmitted effects.

Declaration of interest

The authors declare that they have no competing interests. This work was supported by the Centre of Pharmaceutical Nanoscience and Nanotoxicology (CPNN) financed by the Danish Council for Independent Research. The Danish Council for Independent Research (Grant no. 12-126262) and by the Lundbeck Foundation Center for Biomembranes in Nanomedicine (CBN).

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Supplementary material available online
Supplementary S1–S9.