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Expression of adhesion molecules, monocyte interactions and oxidative stress in human endothelial cells exposed to wood smoke and diesel exhaust particulate matter

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ABSTRACT

Toxicological effects of wood smoke particles are less investigated than traffic-related combustion particles. We investigated the effect of wood smoke particles, generated by smouldering combustion conditions, on human umbilical endothelial cells (HUVECs) co-cultured with or without monocytic THP-1 cells. Standard reference material (SRM) 2975 diesel exhaust particles were used as benchmark particles. Wood smoke particles at 50 μg/ml or 100 μg/ml caused adhesion of THP-1 monocytes onto HUVECs in co-cultures, whereas SRM2975 had no such effect. Both types of particles from 1 μg/ml increased VCAM-1 expression on HUVECs in mono-cultures. However, only the exposure to wood smoke particles was associated with increased expression of *TNF* and *IL8* mRNA in THP-1 cells. We found no effect on the intracellular production of reactive oxygen species by the fluorescent probe DCFH-DA, whereas especially the wood smoke particles caused increased level of DNA strand breaks and oxidised guanines at concentrations with low cytotoxicity. In conclusion, our results indicate that the adherence of monocytes on endothelial cells in wood smoke particle exposed cultures depend on activation of both cell types.

1. Introduction

Combustion of wood for heating and cooking emits a substantial part of the particles found in ambient air. However, the toxicological effects of wood smoke particles (WPs) are still less investigated than the effect of traffic-generated combustion particles. Recent studies have shown that exposure to WPs is associated with elevated production of reactive oxygen species (ROS), glutathione depletion, heme oxygenase-1 (HO-1) upregulation, increased expression and release of inflammatory cytokines, and increased levels of oxidatively damaged DNA bases in lung and monocyte related cell cultures (Danielsen et al., 2009, 2011; Kocbach et al., 2008a; Liu et al., 2005). In these studies, smouldering combustion conditions generated particularly large amounts of particles with high toxicity. Airway and gastrointestinal tract exposure to WPs also caused inflammation in the lungs and liver of rats, and the latter was accompanied by DNA adducts from lipid peroxidation products and *HO-1* upregulation (Danielsen et al., 2010). These effects – oxidative stress and inflammation – are also implicated in the development of endothelial dysfunction, which plays an important role in the progression of atherosclerosis (Donaldson et al., 2001; Madamanchi et al., 2005). The endothelium functions as a barrier and it produces factors regulating the vascular tone, cellular adhesion, and vessel wall inflammation (Carter and Wicks, 2001; Madamanchi et al., 2005). Endothelial cells, activated during inflammation, express intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the membrane (Carlos and Harlan, 1994; Carter and Wicks, 2001). These cell surface molecules mediate the firm binding of leukocytes on the endothelial cells and transmigration of monocytes across the endothelium (Videm and Albrigtsen, 2008). This recruitment of inflammatory cells is important both for the atherosclerosis process and lung inflammation.

The aim of this study was to investigate the effect of WPs and standard reference material (SRM) 2975 on activation of endothelial cells and interactions with monocytes with the use of mono- and co-cultures of human umbilical vein endothelial cells (HUVECs) with the THP-1 monocytic cell line. We hypothesised that exposure to particles stimulates the adherence of monocytes on endothelial cells by effects that are related to oxidative stress and inflammation. HUVECs were chosen because they can be activated to express

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adhesion molecules and thereby promote adherence of monocytes by exposure to particulate matter (Montiel-Davalos et al., 2007). The THP-1 cells can be transformed to macrophages, which is a relevant mechanistic event in atherogenesis where early plaques are characterised by recruitement of monocytes and differentiation to foam cells that have characteristics similar to macrophages. We measured the expression of ICAM-1 and VCAM-1 because these cell surface adhesion molecules are used experimentally as early markers of vascular diseases and because they are important for recruitment of inflammatory cells into the lung. Oxidative stress effects were assessed as the reactive oxygen species (ROS) production detected by a fluorescent probe within the cells and the level of oxidatively damaged DNA base lesions in the endothelial cells measured by the comet assay. The level of oxidatively damaged DNA is a widely used biomarker of oxidative stress in human studies of exposure to particulate matter (Møller and Loft, 2010) and it is also considered to have predictive value in regard to risk of lung cancer in prospective studies (Loft and Møller, 2006; Loft et al., 2006, 2012). The inflammatory activation of THP-1 cells was assessed by cytokine gene expression. The effects of WPs collected under smouldering combustion conditions were compared to those of SRM2975 as combustion-derived benchmark diesel exhaust particles (DEP), which are well-characterised and investigated in multiple studies on particle-induced inflammation and oxidative stress (Møller et al., 2010).

2. Materials and methods

2.1. Cell lines

HUVECs and culture medium were purchased from Cell Applications (San Diego, CA, USA). The cells were cultured in Endothelial Cell Growth Medium Kit, which contained 2% serum, at 37 °C in 5% CO_2–95% air gas mixtures. The medium was changed 24–36 h after seeding and the cells were cultured until they were 90% confluent. The cells were used between passages 2 and 6, because they maintain morphologic and phenotypic characteristics as a primary endothelial cell in these passages. The monocytic cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI with 10% serum as previously described (Danielsen et al., 2009). The THP-1 cell line is a suspension cell, but upon activation with phorbol 12-myristate 13-acetate (PMA) the cells are activated to differentiate into macrophages and becomes adherent.

2.2. Particles

The WPs were collected over a 2 h combustion period of beech wood (water content 12–18%) with low oxygen supply in the stove (Model 7110, Morsø, Nykøbing Mors Denmark) as previously described (Danielsen et al., 2011). The wood was combusted in logs of approximately 1 kg at temperatures between 30 and 60 °C in the flue gas, mostly below 50 °C. These smouldering conditions represent a poor combustion of the wood and the particulate matter (PM) therefore represents potentially more hazardous material than PM generated by optimal combustion conditions (Kocbach et al., 2009). Levels of PAH and soluble metals were determined as previously reported (Danielsen et al., 2011). The endotoxin concentration was assessed by Pyrogent Gel Clot LAL Assay, which had a sensitivity of 0.06 EU/ml (Danielsen et al., 2011). We used SRM2975 as benchmark particle because there are no reference particles for wood smoke exposure.

The SRM2975 was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The WPs and SRM2975 should not be regarded as having the same particle characteristics, except that they are both combustion-derived particles.

The PM stock solution was prepared by sonicating a 1 mg/ml suspension of particles in cell culture medium or Hanks balanced salt solution (Sigma–Aldrich), depending on the assay, using a Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a disrupter horn (Model number: 101-147-037) before each experiment. The exposure media were sonicated for 8 min with alternating 10 s pulses and 10 s pauses at amplitude of 10% and continuously cooling on ice to avoid sample heating and evaporation. The size distribution of particles in the exposure cell culture media was determined by the Nanosight optical tracking system (NTA; Nanosight LM20, Amesbury, Wiltshire, United Kingdom). It visualises and analyses particles in liquids from 10 to 1000 nm using Brownian motion and random migration models to calculate diffusion coefficients and subsequently the Stokes–Einstein's equation is utilised to determine particle size as hydrodynamic diameter.

2.3. Cell adhesion assay

The adhesion was assessed by measurement of 5-bromo-2′-deoxyuridine (BrdU)-labelled THP-1 cells onto HUVECs. We seeded 2.5×10^4 HUVECs/well in 96 well plates in 200 μL media. After overnight culture, the media were removed and 5×10^3 THP-1 cells labelled with BrdU (Roche, Germany) were added to each well and cultured for 24 h with the HUVECs. The co-cultures were exposed to particles in a concentration-dependent manner as well as to the positive controls TNF (100 ng/ml) and PMA (100 ng/ml) for another 24 h. After exposure, the media were removed and the cells were rinsed twice with 100 μL PBS. The media and PBS were transferred to another 96 well plate. After centrifugation the BrdU content both in the co-culture and supernatant were determined according to the manufactures instructions (Roche, Germany). In brief, the media were removed and the cells were fixed. The antibody solution was added for binding with BrdU-labelled DNA. After washing three times, the immune complexes were detected by *tert*-methylbenzidine. The reaction was stopped with 50 μL 2 M HCl and the absorbance was determined by an ELISA-reader at 450 nm with 690 nm as reference. To further study the attachment of THP-1 cells to Petri dishes, 5×10^3 THP-1 cells labelled by BrdU were also exposed without HUVECs and the attachment of THP-1 cells was analysed by the method above. The percentage of BrdU in the co-culture and supernatant was calculated as follows:

$$\text{Percentage}\ (\%) = \frac{\text{BrdU OD culture}}{\text{BrdU OD culture} + \text{BrdU OD supernatant}} \times 100$$

The cell–cell interaction was also assessed by visual inspection of the co-cultures as the number of THP-1 cells attached onto the layer of HUVECs. HUVECs (3×10^5 cells/well) were seeded in a 6 well plate and cultured to near confluence (3–4 days). After this 1.5×10^5 THP-1 cells were added with different exposure conditions (control, 50 μg/ml of SRM2975 or WPs, 100 ng/ml of TNF or PMA) and incubated for 24 h at 37 °C. Subsequently, the medium was removed and the cells were washed three times. Digital images of the cell were obtained with 20 times magnification. The extent of adhesion of THP-1 cells on HUVECs was assessed by visual scoring of the coded pictures; two persons, who were unaware of the codes, scored the pictures in quadruplicates on four independent days.

2.4. Measurement of ICAM-1 and VCAM-1

The surface expression of ICAM-1 and VCAM-1 was measured by a modified ELISA procedure (Rasmussen et al., 2001). HUVECs (2×10^4 cells/well) were seeded in a 96-well plates and left overnight for proper attachment. The next day, freshly prepared particle suspensions were added to the cells in concentrations of 0, 1, 10, 50 and 100 μg/ml and incubated for 24 h. Following this, the cells were incubated with anti-ICAM-1 (cat. no. BBA17, R&D Systems, Abingdon, UK) or anti-VCAM-1 (cat. no. BBA19, R&D) in a 1:500 dilution for 1 h at 37 °C. Subsequently, the plates were washed three times with warm medium with 1% BSA and placed on ice. The secondary antibody, anti-goat IgG peroxidase coupled antibody (cat. no. A5420, Sigma, MI, USA) was diluted 1:25 000 with PBS containing 0.1% Tween (PBS/Tween) and incubated with the cells for 1 h on ice. The plates were then washed five times with ice-cold PBS/Tween. A substrate solution containing one *o*-phenylenediamine (OPD) tablet (Sigma) was dissolved in phosphate citrate buffer (phosphor citrate buffer tablets, Sigma) and H_2O_2 was added immediately before use to a final concentration of 3.5 mM. This substrate solution was added to the wells and incubated for 30 min in the dark at room temperature. The absorbance was measured in a Multiskan Ascent reader (Labsystems) at 450 nm. The pro-inflammatory cytokine TNF (0, 0.1, 1, 10, and 100 ng/ml) was used as a reference control. The data were corrected for background and particle interference.

2.5. Cytotoxicity

The cytotoxicity of the test samples was measured using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. Briefly, HUVECs (2×10^4 cells/well) were seeded in 96-well plates and cultured for 24 h. They were then incubated with suspensions of particles in Endothelial Cell Growth Medium (0, 1, 2.5, 10, 25, and 100 μg/ml) for 24 h. Then, the culture media (supernatant) were collected and mixed with reaction buffer consisting of catalyst (diaphorase/NAD^+ mixture) and dye solution (iodotetrazolium chloride and sodium lactate). After 1 h of incubation the absorbance, corresponding to the level of lactate dehydrogenase (LDH) release, of the samples was measured at 490 nm using an ELISA reader (Labsystems, Multiskan Ascent). Maximum LDH content of the HUVECs was assessed by treating HUVECs with 1% Triton X-100 solution, and cytotoxicity of the test samples was expressed as the fraction of LDH activity relative to this maximum LDH activity.

To ensure that the primary particle suspensions did not interfere with the Cytotoxicity Detection Kit, particle suspensions were added to control wells without cells and incubated overnight like the rest of the samples. The data were corrected for background and particle interference. The experiments were performed in triplicates on three independent days.