Investigating the effects of cerium dioxide nanoparticles and diesel exhaust co-exposure to the epithelial airway barrier at the air-liquid interface *in vitro*

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Cerium dioxide (CeO_2) nanoparticles (NPs) are currently being used as a fuel additive to assist catalytic regeneration of diesel particle filters and to promote more efficient fuel consumption[1]. Although particles emitted from diesel engines (DEPs) are known to be carcinogenic[2], the effects of exhaust emissions containing DEPs+CeO_2 NPs upon human respiratory health are not fully understood. The aim of this study therefore, was to investigate the effects of a combined exposure of DEPs+CeO_2 NPs on cell cultures *in vitro.*

A sophisticated 3D *in vitro* model of the human epithelial airway barrier[5], composed of (i) a confluent epithelial cell layer of 16HBE14o$^-$ cells, (ii) human whole blood monocyte derived macrophages (MDM) and (iii) human whole blood monocyte dendritic cells (MDDC) was used. To further reflect the physiological conditions *in vivo* this triple cell co-culture system was cultured at the air-liquid interface, as previously described in Blank *et al*. (2007). The use of this *in vitro* system is advantageous over alternative *in vitro* systems, such as monocultures, since it combines both the physical barrier of epithelial cells and two important immune cells (MDM and MDDC). For exposure to DEPs, a well established exposure system[4] was used in which cell cultures can be exposed to freshly produced diesel exhaust under controlled conditions. As a test vehicle, an Opel Astra X20DTL without exhaust after treatment system was used. The vehicle was running on a dynamometer at constant velocity of 35km/h (engine speed 2180rpm) with low sulfur diesel (Greenergy SA, Steinhausen, Switzerland) and normal lubrication oil (Motorex). The force at the wheel was set to 66N.

In parallel to the exposure experiments, the exhaust was analyzed by measuring the elemental carbon mass with a photoelectric aerosol sensor (PAS), the total particles surface with a diffusion charging particle sensor (DC), the number of particles and the distribution of particles between 10-400nm with an electrostatic classifier with differential mobility analyser (DMA) in combination with a condensation particle counter. Before brought into contact with the cells, exhaust samples were diluted 1:10 with absolute clean air, heated to 37℃, and brought to a relative humidity of 85% and a CO_2 concentration of 5%. The volume flow was set to 2l/min.

For exposure to DEPs, the cells were kept in the exposure system for 2 hours or 6 hours, reflecting a low and a high dose. For co-exposures to DEPs+CeO_2 NPs, an aerosolized solution of CeO_2 NPs was nebulized over the cell culture surface after the exposure to diesel exhaust. Cell cultures that had been exposed to low does of DEPs were exposed to 20µg/ml solution, the ones that had been exposed to high doses of DEPs to 60µg/ml solution. Upon CeO_2 NP exposure, the cultures were incubated for 6 hours at 37℃, 85% relative humidity and 5% CO_2. To be able to differentiate between effects of DEPs, CeO_2 NPs and DEPs+CeO_2 NPs, one set of cell cultures was exposed to DEPs only, one set to CeO_2 NPs only and one set to DEPs+CeO_2 NPs. To further be able to differentiate between effects of DEPs and DEPs independent effects of the conditions in the exposure system, additional sets were exposed to absolute pure air (37℃, 85% relative humidity, 5% CO_2) instead of DEPs. After incubation, the cells and the cell culture media were collected for the measurement of biological responses.

To estimate cytotoxic effects of the exposures, the release of lactate dehydrogenase (LDH) into the culture medium was quantified. LDH is a cytosolic enzyme, which when released into the extracellular media is a predictor for the permeability of the cellular membrane, indicative of a cytotoxic response. Additionally, fluorescent microscopy of cell cultures labelled for nuclear DNA content and the actin cytoskeleton was performed to assess any morphological changes to the epithelial cell layer following exposure to diesel exhaust. The ability for diesel exhaust to cause oxidative stress *in vitro* was assessed via quantification of total reduced glutathione (GSH), a cellular thiol involved numerous cellular processes, such as the scavenging of reactive oxygen species and the protection of cellular components. In addition, the expression of superoxide-dismutase 1 (SOD1) and heme-oxygenase 1 (HO1) by real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR) was assessed. (Pro)-Inflammatory responses were estimated by the quantification of the release of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α and chemokine interleukin (IL)-8 into the culture medium by enzyme linked immunosorbent assay (ELISA) and by measurement of their

expression levels via real-time RT-PCR. The ability for all exposures to cause apoptosis was assessed by measuring the expression levels of caspase7 and FAS by real-time RT-PCR.

The quantification of extracellular LDH showed that DEPs, CeO_2 NPs and DEPs+CeO_2 NPs elicit no significant cytotoxicity, independently of the dose. The same was found by microscopy, which did not reveal any changes in cellular morphology or in the density of the epithelial layer. Quantification of total reduced GSH showed that DEPs decreases the amount of antioxidant molecules in the cell to less than 5% of the controls. This effect was statistically significant ($p<0.05$) and independent of CeO_2 NPs or the dose. Despite this drastic drop in the cellular antioxidant capacity, the expression level of SOD1 did not show significant changes. HO1 expression increased significantly ($p<0.05$) upon exposure to low doses of DEPs. This effect was abolished by CeO_2 NPs, whereas low doses of CeO_2 NPs alone did not have an effect. High doses of DEPs and CeO_2 NPs both resulted in a significant ($p<0.05$) increase of HO1 expression and had a synergistic effect upon co-exposure. This synergistic effect of the two particle types was statistically significant ($p<0.05$). A significant change in expression levels of TNF-α and IL-8 was not detected, independently of the dose and the exposure type. Also, the quantification of the release of the cytokines showed no significant effects. However, a clear dose dependent tendency of elevated TNF-α release upon exposure to DEPs was observable, an effect which was independent of CeO_2 NPs. Significant apoptotic responses were not detectable independently of the dose and the exposure type. However, for FAS, a tendency towards lower expression was observable independently the exposure type but dependent on the dose. Caspase7 showed a tendency towards lower expression upon high dose exposure to CeO_2 NPs and DEPs+CeO_2 NPs, but upon exposure to high doses of DEPs alone.

In summary, the observation that even the highest doses of DEPs and CeO_2 NPs used did not affect cell viability or morphology indicates that the measured responses can be considered as the result of an intact cellular regulatory network. DEPs were found to exert a highly oxidative effect on human respiratory epithelial cells *in vitro*, an effect which is not influenced by the presence of CeO_2 NPs. The resulting transcriptional activation of oxidative stress-responsive genes however, is influenced by CeO_2 NPs in dose dependent and gene specific manners and synergistic effects of the two particles are observable. An induction of a pro-inflammatory response is observable upon exposure to DEPs but this induction is not statistically significant. The extent of induction is dependent on exposure duration and independent on the presence of CeO_2 NPs. Significant apoptotic responses are not induced. There is however a trend towards lower expression of pro-apoptotic genes upon exposure to DEPs and CeO_2 NPs for FAS and tendency towards lower expression of caspase7 upon exposure to high doses of

CeO_2 NPs observable. In conclusion, CeO_2 NPs do not influence the primary toxicity of DEPs, but they cause changes in the cellular responses and might thereby influence secondary toxicity.

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4 Muller *et al*. (2010); *Environ Sci Technol*, **44:** 2632-2638.

Effect of CeO_2 Nanoparticles on Diesel Exhaust Toxicity

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Introduction

Cerium dioxide nanoparticles (CeO_2 NPs) are used in fuel additives to assist catalytic regeneration of diesel particle filters and to render fuel consumption more efficient[1,2]. Although the effects of particles emitted from diesel engines (DEPs) upon human health are well described[3,4], the effects of exhaust emissions containing DEPs+CeO_2 NPs are poorly understood. The aim of this study was therefore to investigate how CeO_2 NPs in diesel exhaust affect DEP toxicity on a 3D triple cell co-culture model of the human epithelial airway barrier at the air-liquid interface *in vitro*[5], using a well established vehicle exhaust exposure system[6].

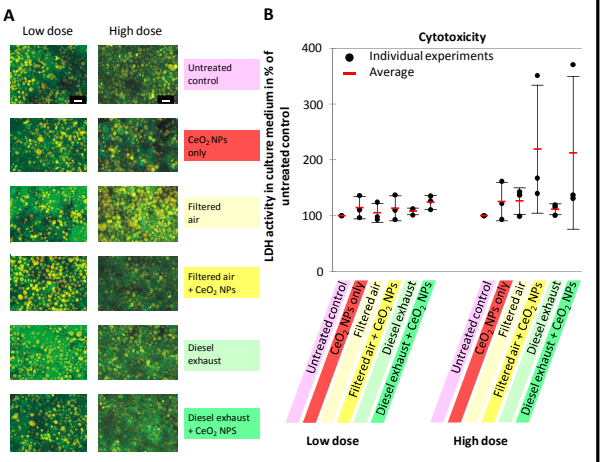
Experimental procedure

Triple cell co-cultures composed of the bronchial epithelial cell line 16HBE14o$^-$ and human monocyte derived dendritic cells and macrophages were exposed to diluted diesel exhaust (4.75x10^8 particles/cm^3, 2l/min) for two hours (low dose) or 6 hours (high dose). Upon diesel exhaust exposure, the cell cultures were sprayed with 50µl of aerosolized CeO_2 NP solution (20µg/ml for the low dose and 60µg/ml for the high dose). Biological responses were measured after 6 hours post-incubation

Results

Cellular morphology and cytotoxicity

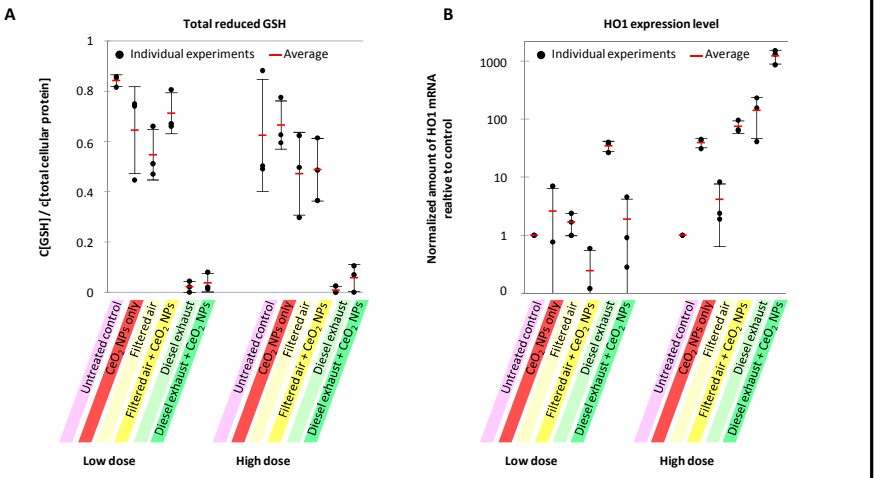
A: Cellular morphology was assessed by microscopy of cell cultures fluorescently labeled for actin (green) and nucleic acids (yellow). Scale bars represent 25µm. **B**: cytotoxicity was estimated by quantification of lactate dehydrogenase (LDH) in the culture medium. Error bars indicate standard deviations, n=3.



Cellular morphology is not affected by exposure to DEPs and/or CeO_2 NPs. Exposure to high doses of CeO_2 NPs and DEPs+CeO_2 NPs resulted in slightly increased cytotoxicity.

Oxidative stress and according transcriptional responses

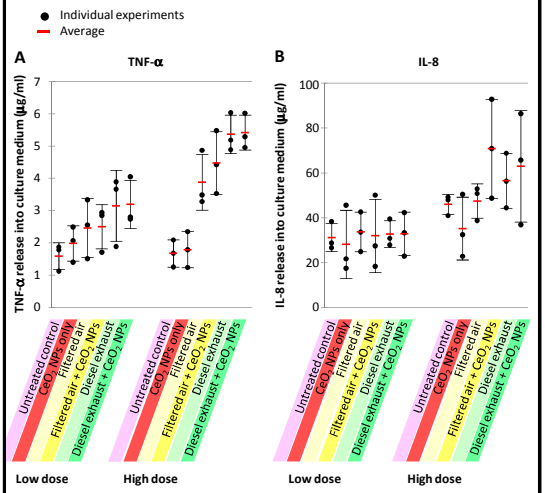
A: The depletion of the cellular pool of antioxidant molecules was estimated by quantification of total reduced glutathione (GSH). Cellular responses to oxidative stress were estimated by measurement of transcriptional induction of **B**: heme-oxygenase 1 (HO1) and superoxide-dismutase 1 (SOD1, data not shown) by real-time RT-PCR. Error bars indicate standard deviations, n=3.



The oxidation of GSH is due to DEPs, with CeO_2 NPs having only small effects. Transcriptional activity of HO1 is reduced by low, and increased by high doses of CeO_2 NPs. SOD1 shows weak responses, independently of particle type and dose (data not shown).

Pro-inflammatory responses

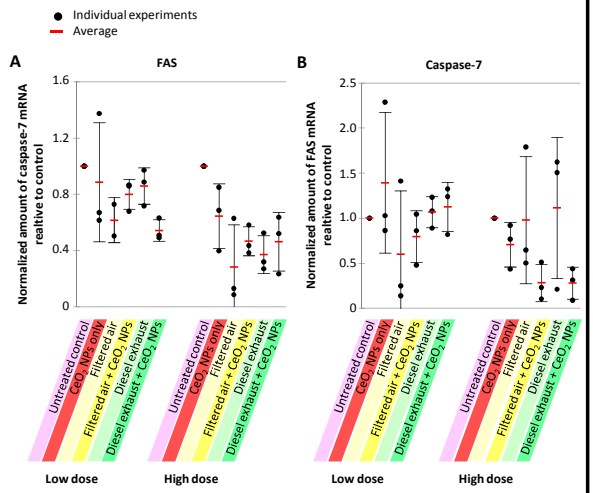
Pro-inflammatory responses were estimated by quantification of **A**: tumor necrosis factor (TNF)-α and **B**: interleukin (IL)-8 in the culture medium by ELISA. Error bars indicate standard deviations, n=3.



The induction of pro-inflammatory responses is mainly caused by DEPs alone and is dose dependent.

Apoptosis

Stimulation of apoptosis was assessed by measurement of the transcriptional activity of **A**: caspase-7 and **B**: FAS by real-time RT-PCR. Error bars indicate standard deviations, n=3.



Both particle types decrease the transcriptional activity of pro-apoptotic genes in a dose dependent manner.

Summary and conclusion

Whereas the induction of oxidative stress relies mainly on DEPs, CeO_2 NPs play a role in the induction of the according transcriptional response. Pro-inflammatory responses are mainly induced by DEPs in a dose dependent manner. Both particle types are able to decrease the transcriptional activity of pro-apoptotic genes.

Our results indicate that CeO_2 NPs have little effect on the primary toxicity of diesel exhaust but cause modulations in the cellular responses to diesel exhaust exposure.

Materials and methods:

Monocyte isolation was performed by CD14 positive selection (CD14 MicroBeads, Miltenyi Biotec). Monocyte differentiation and preparation of the triple cell co-cultures is described in *(5)*, the exposure system in *(6)*, the preparation of the CeO_2 NPs in *(7)*. Determination of extracellular LDH concentration: LDH Assay Kit, Roche Applied Sciences; extracellular GSH: Glutathione Kit, Cayman Chemical; TNF-α and IL-8: human TNF-alpha DuoSet and human CXCL8/IL-8 DuoSet, R&D Systems; Real-time RT-PCR: RT: Omniscript Reverse Transcriptase (Qiagen), real-time PCR: Fast SYBR Green Mastermix (Applied Biosystems), 7500 Fast Real-Time PCR System (Applied Biosystems)

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