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 (CeO₂) nanoparticles (NPs) are currently being used as a fuel additive to assist catalytic regeneration of diesel particle filters and to promote more efficient fuel consumption¹. Although particles emitted from diesel engines (DEPs) are known to be carcinogenic², the effects of exhaust emissions containing DEPs+CeO₂ 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+CeO₂ NPs on cell cultures in vitro.

A sophisticated 3D in vitro model of the human epithelial airway barrier⁵, 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 system⁴ 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°C, and brought to a relative humidity of 85% and a CO₂ 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+CeO₂ NPs, an aerosolized solution of CeO₂ NPs was nebulized over the cell culture surface after the exposure to diesel exhaust. Cell cultures that had been exposed to low doses of DEPs were exposed to 20µg/ml solution, the ones that had been exposed to high doses of DEPs to 60µg/ml solution. Upon CeO₂ NP exposure, the cultures were incubated for 6 hours at 37°C, 85% relative humidity and 5% CO₂. To be able to differentiate between effects of DEPs, CeO₂ NPs and DEPs+CeO₂ NPs, one set of cell cultures was exposed to DEPs only, one set to CeO₂ NPs only and one set to DEPs+CeO₂ 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°C, 85% relative humidity, 5% CO₂) 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-$\alpha$ 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-$\alpha$ 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\textsubscript{2} NPs observable. In conclusion, CeO\textsubscript{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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Effect of CeO₂ Nanoparticles on Diesel Exhaust Toxicity

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Introduction

Cerium dioxide nanoparticles (CeO₂ NPs) are used in fuel additives to assist catalytic regeneration of diesel particle filters and to render fuel consumption more efficient². Although the effects of particles emitted from diesel engines (DEPs) upon human health are well described⁴, the effects of exhaust emissions containing DEPs+CeO₂ NPs are poorly understood. The aim of this study was therefore to investigate how CeO₂ 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 vitro⁵, using a well established vehicle exhaust exposure system⁶.

Experimental procedure

Triple cell co-cultures composed of the bronchial epithelial cell line 16HBE14o-1 (high dose) upon diesel exhaust exposure, the cell cultures were sprayed with 50μl of aerosolized CeO₂ 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

Pro-inflammatory responses

Stimulatory effects of CeO₂ 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 Materials and methods. Rutishauser et al. (2005) and Kasper et al. (2006) provided the human lung epithelial cell lines and the human monocytes. Exposure to CeO₂ NPs alone in the low dose range (0.4-1 μg/ml) or in the high dose range (4-10 μg/ml) did not affect cellular morphology, as assessed by microscopy of cell cultures fluorescently labeled for actin (green) and nucleic acids (yellow). Scale bars represent 25 μm. Cellular morphology was not affected by exposure to DEPs and/or CeO₂ NPs. Exposure to high doses of CeO₂ NPs and DEPs+CeO₂ NPs resulted in slightly increased cytotoxicity.

Oxidative stress and according transcriptional responses

The induction of pro-inflammatory responses is mainly caused by DEPs alone and is dose dependent. Both particle types decrease the transcriptional activity of pro-apoptotic genes in a dose dependent manner. The induction of oxidative stress relies mainly on DEPs, CeO₂ 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.

Summary and conclusion

Our results indicate that CeO₂ NPs have little effect on the primary toxicity of diesel exhaust but cause modulations in the cellular responses to diesel exhaust exposure.

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Summary and conclusion

Whereas the induction of oxidative stress relies mainly on DEPs, CeO₂ 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 CeO₂ NPs have little effect on the primary toxicity of diesel exhaust but cause modulations in the cellular responses to diesel exhaust exposure.