



Reduction in (pro-)inflammatory responses of lung cells exposed *in vitro* to diesel exhaust treated with a non-catalyzed diesel particle filter[☆]

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HIGHLIGHTS

- A non-catalyzed DPF decreases the oxidative potential of diesel exhaust.
- Inhalation of diesel exhaust induces severe oxidative stress *in vitro*.
- Unfiltered diesel exhaust induces acute pro-inflammatory responses *in vitro*.
- Filtered diesel exhaust does not induce acute pro-inflammation *in vitro*.
- Exhaust filtration alone is not sufficient to reduce *in vitro* diesel exhaust toxicity.

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ABSTRACT

Increasingly stringent regulation of particulate matter emissions from diesel vehicles has led to the widespread use of diesel particle filters (DPFs), the effect of which on exhaust toxicity is so far poorly understood. We exposed a cellular model of the human respiratory epithelium at the air-liquid interface to non-catalyzed wall-flow DPF-filtered diesel exhaust and compared the resulting biological responses to the ones observed upon exposure to unfiltered exhaust. Filtered diesel exhaust acted highly oxidative, even though to a lesser extent than unfiltered exhaust (quantification of total reduced glutathione), and both exhaust types triggered comparable responses to oxidative stress (measurement of heme-oxygenase 1 (*HMOX1*) and superoxide-dismutase (*SOD1*) gene expression). Further, diesel exhaust filtration significantly reduced pro-inflammatory responses (measurement of tumor necrosis factor (*TNF*) and interleukin-8 (*IL-8*) gene expression and quantification of the secretion of their gene products *TNF-α* and *IL-8*). Because inflammatory processes are central to the onset of adverse respiratory health effects caused by diesel exhaust inhalation, our results imply that DPFs may make a valuable contribution to the detoxification of diesel vehicle emissions. The induction of significant oxidative stress by filtered diesel exhaust however, also implies that the non-particulate exhaust components also need to be considered for lung cell risk assessment.

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1. Introduction

The percentage of diesel cars in the total passenger car fleet in Western Europe grew from 13.8% in 1990 to 50.6% in 2010 (ACEA, European automobile manufacturers' association. Resulting in considerably higher traffic-related emissions of nitrogen oxides (NO_x), hydrocarbons (HC) and particulate matter (Chan et al., 2007). Of special interest is the particulate fraction of diesel exhaust, the

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diesel exhaust particles (DEPs). Because of their small average diameter of 60–100 nm (Bartscher, 2005), DEPs are readily inhaled and deposit on the lung surface where they are retained for prolonged time periods (Moller et al., 2008). Via the generation of reactive oxygen- and nitrogen species (ROS/RNS), interactions of the deposited DEPs with the human respiratory tract may result in the induction of oxidative stress and the subsequent induction of (pro-)inflammatory responses (Donaldson et al., 2005; Reuter et al., 2010; Xiao et al., 2003). Oxidative stress induced inflammatory responses lead to the formation of even more ROS and RNS (Reuter et al., 2010), which may result in the local induction of apoptosis (Simon et al., 2000), chronic lung diseases (Donaldson et al., 2005), allergic lung inflammation (Nel et al., 1998) and oxidative DNA damage (Schins and Knaapen, 2007) and may also cause systemic effects (Donaldson et al., 2005; Polichetti et al., 2009).

Considering the vast impact of DEPs on human health, the importance of reducing particle emissions from diesel engines is evident. This is reflected in increasingly stringent exhaust emission legislation. Whereas in the Euro I regulation for diesel passenger cars, the limit value for particle mass (PM) emission was set to 140 mg km^{-1} (Council directive 70/156/EEC and 91/441/EEC), with the Euro 6 regulation the PM limit value is set to 4.5 mg km^{-1} for all passenger cars and to 6×10^{11} particles/km for diesel passenger cars (Regulation (EC) No 715/2001 and 692/2008). The introduction of a limit value for the particle number takes into account that particle mass is not sufficient for estimating the biological impact resulting from particle exposure (Oberdorster et al., 1994).

The key technology to reduce particle emissions of diesel engines are diesel particle filters (DPFs). Among those, wall flow filters, reaching mass filtration efficiencies higher than 99% (Mayer et al., 2010), are the most common ones. In wall-flow DPFs, exhaust gas is forced through a porous wall in which particles but not gases are retained. In order to keep the resulting backpressure over the filter low, the filter has to be regenerated, i.e. the accumulated particles are oxidized, either by engine-initiated processes (active regeneration, e.g. fuel post-injection) or continuously (passive regeneration, e.g. the continuous regeneration trap). In order to assist the oxidation of the accumulated particles, DPFs may be catalytically coated or operated in combination with catalysts, e.g. a diesel oxidative catalyst (van Setten et al., 2001). In particular when operated in combination with catalytic systems, DPFs have profound effects on exhaust composition besides removing particulate matter. For instance, removal but also *de-novo* formation of polyaromatic hydrocarbons (PAHs) and nitro-PAHs and in rare cases polychlorinated dibenzo-dioxins has been reported, (Heeb et al., 2010, 2007).

Till today, only very few studies have been performed on how these changes in exhaust composition affect exhaust toxicity and to the best of our knowledge, no studies investigating effects of a non-catalyzed DPF on the *in vitro* toxicity in lung cells of diesel exhaust have been conducted so far under realistic experimental settings. It is therefore hardly known what contribution to the overall exhaust toxicity is made by the particulate fraction, which is however, important to know when designing future exhaust after-treatment systems.

The aim of the present study was to compare the toxicity of unfiltered and filtered diesel exhaust. In order not to considerably change any parameters of the tested exhaust other than the particle content, a non-catalyzed DPF was used for exhaust filtration. A three-dimensional model of the human airway barrier consisting of epithelial cells, macrophages and dendritic cells (Blank et al., 2007; Rothen-Rutishauser et al., 2008, 2005) was exposed to unfiltered or filtered diesel in an air-liquid cell exposure system (Muller et al., 2010). This reflects the situation *in vivo* and, by reducing the interaction between exhaust and culture medium, minimizes the

risk of generating artefacts (Muller et al., 2011). Samples of complete engine exhaust were taken directly at the tailpipe with the exhaust characterization being performed in parallel to the exposure experiments, thereby making sure that no unwanted exhaust aging effects or biased sample generation occurred.

2. Materials and methods

2.1. Cell cultures

A 3D *in vitro* model of the human epithelial airway barrier (Blank et al., 2007; Rothen-Rutishauser et al., 2008, 2005) was used as a biological system. A detailed description of how the cell cultures were produced is given in the Supporting Information. Briefly, a confluent layer of 16HBE14o⁺ epithelial cells was cultured on an insert membrane. Human whole blood monocyte derived macrophages were cultured on the apical side and human whole blood monocyte-derived dendritic cells on the basal side. Depart from the protocol provided by Blank et al., (2007), human whole blood monocytes were isolated from buffy-coats using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's manual, which considerably increases the purity of the monocytes.

2.2. Test vehicle and exposure system

The test vehicle was an Opel Astra X20DTL (registration in 1998, in Switzerland, mileage in course of the experiments: 70,000–75,000 km) running on a dynamometer at a constant velocity of 35 km/h^{-1} (corresponding to an engine speed 2180rpm) with a force of 66N at the wheel. The vehicle was operated with commercially available low sulfur diesel ($<10 \text{ mg kg}^{-1}$, Greenergy SA) and the recommended lubrication oil (V10.237, Motorex). Exhaust was sampled at the tailpipe and after ten-fold dilution with filtered ambient air (air temperature: 120°C . Type H filter, MSA. In the filtered air, no particles could be detected), and it reached the cells with a delay of less than 30 s. The exhaust sample temperature had been adjusted to 37°C at this point.

All parts of the originally installed exhaust after-treatment system were removed from the test vehicle, so that the exhaust could be considered as an engine-out emission (unfiltered exhaust). Filter regeneration took not place during the exposure experiments. For experiments with filtered exhaust, a silicon carbide DPF (IBIDEN, 200 cpsi, pore size $10 \mu\text{m}$ with narrow pore-size distribution (Ogyu et al., 2006)), obtained from a Peugeot 406 was installed at the tailpipe. The oxidative catalyst had been removed in order not to change any parameters of the exhaust composition other than the particulate fraction. This does not affect the filtration efficiency of the filter. After 3 days of operation (824 km), the DPF was removed and regenerated, followed by three further days of operation (791 km). After regeneration, the filter was loaded until the particle number-size distribution downstream of the DPF was stable again prior to further exposure experiments. The exposure system used (a scheme is given in Supporting Information Figure S1) has previously been described in detail (Morin et al., 2008; Muller et al., 2010). Diluted exhausts were brought to the exposure chambers with volumes of 4 L per chamber at a constant volume flow of 2 L min^{-1} . Throughout the exposures, conditions within the exposure chambers were maintained at 37°C , 5% CO_2 and 80% relative humidity.

2.3. Exhaust characterization

The Exposure experiments and hence the online exhaust characterization were performed at the exhaust gas control station of the Bern University of Applied Sciences in Nidau, Switzerland, an

institution officially accredited for exhaust gas control by the Swiss government. All measurements have been performed by qualified personnel according to the requested protocols.

The size-number distribution, the elemental carbon (EC) content and the total active surface area of the particulate exhaust fraction were measured in the ten-fold diluted exhaust using a scanning mobility particle sizer (differential mobility analyzer: TSI 3081; condensation particle counter: TSI 3772 A), a photoelectric aerosol sensor (EcoChem PAS, 2000) and a diffusion charging sensor (Matter Engineering LQ1-DC) respectively. Furthermore, the concentrations of carbon monoxide (CO), total gaseous hydrocarbons (HC), nitrogen oxides (NO_x) and nitrogen monoxide (NO) were measured in the ten-fold diluted exhaust using the Horiba MEXA-9400H exhaust gas measuring system. Concentrations of nitrogen dioxide (NO₂) were estimated based on the assumption that NO_x is entirely made up of NO and NO₂.

2.4. Exposure conditions

The cell cultures were exposed at the air-liquid interface for 2 or 6 h in order to reflect a low and a high dose situation. All cultures were then post-incubated for 6 h at 37 °C, 5% CO₂ and 80% relative humidity. These exposure conditions are based on previous experiments (Muller et al., 2010). In each experimental repetition, identical cell cultures (produced with immune cells from the same blood donor) were exposed to diluted diesel exhaust or filtered ambient air. In addition, in order to assure the general integrity/health of the used culture-batch, an untreated control was kept at the air-liquid interface in the incubator during exhaust/filtered air exposure and post-incubation.

2.5. Particle deposition in the exposure chambers

To count the number of particles per square cm deposited in the exposure system, transmission electron microscopy grids were placed in empty cell culture inserts next to the cell cultures (hence in the same position as the cultures themselves) during the exposure experiments. Deposited particles were counted by transmission electron microscopy as previously described (Muller et al., 2010).

2.6. Laser scanning microscopy

Directly after post-incubation, cell cultures were fixed with paraformaldehyde (3% in PBS pH 7.4). The fixed samples were later stained with phalloidin-rhodamine to label the F-actin cytoskeleton, as well as with 4',6'-diamidino-2-phenylindole (DAPI) to highlight the cell nuclei as previously described (Muller et al., 2010) and subsequently imaged by laser scanning microscopy.

2.7. Cytotoxicity

The release of the cytosolic protein lactate dehydrogenase (LDH) into the culture medium was used to estimate cell membrane integrity and hence cytotoxicity. The total amount of extracellular LDH was quantified using an LDH detection kit (Cytotoxicity Detection Kit, Roche) according to the manufacturer's protocol.

2.8. Quantification of total reduced glutathione

The total amount of reduced glutathione (GSH) in the cell cultures was quantified with a glutathione assay kit (Cayman Chemical). The detailed procedure for cell lysis can be found in the

Supporting Information. The detected concentrations of reduced GSH are reported relative to the concentrations of total protein in the samples, which was quantified using the Pierce BCA Protein Assay kit (Pierce).

2.9. Real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR)

RNA isolation was done with the RNeasy plus kit (Qiagen), the reverse transcriptase reactions with the Omniscript RT system and Oligo dT primers (Qiagen). Real-time PCR was performed in the 7500 fast real-time PCR system, (Applied Biosystems) using Fast SYBR Green master mix (Applied Biosystems) as reporter dye. Relative expression levels of heme-oxygenase (*HMOX1*), superoxide-dismutase (*SOD1*), tumor necrosis factor (*TNF*), interleukin-8 (*IL-8*), caspase7 (*CASP7*) and *FAS* were calculated using the $\Delta\Delta C_t$ method with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal standard gene. A detailed description of the procedure, primer sequences and database accession numbers are listed in **Supporting Information Table S1**.

2.10. Quantification of (pro-)inflammatory cytokines

Tumor necrosis factor (TNF)- α and interleukin (IL)-8 in the culture medium were quantified by enzyme-linked immunosorbent assay (ELISA) using the human TNF- α DuoSet and the human CXCL8/IL-8 DuoSet (R&D Systems) according to the manufacturers' guidelines. All samples were ten-fold diluted for measurement with the required reagent diluent.

2.11. Data analysis and statistics

For each day of exposure (i.e. for each experimental repetition), human blood derived monocytes were isolated from a different buffy-coat – and hence from a different blood donor. Because of the inter-individual variation introduced in this way, relatively high variations in the biological responses between different sets of cell cultures were expected and observed. In order to facilitate the interpretation of the presented data, instead of using the measured absolute values, we normalized all measurements to the filtered air exposure (absolute values are shown in **Supporting Information Table S2**). For comparison between effects of filtered and unfiltered exhaust, we used **formula F1**

$$(\text{EXP}_{\text{DPF}}/\text{REF})/(\text{EXP}_{\text{UNFILTERED}}/\text{REF}) \quad (1)$$

EXP stands for exhaust exposure, REF for filtered air exposure DPF and UNFILTERED for experimental realizations in which filtered exhaust or unfiltered exhaust had been used. Measurements obtained upon exhaust exposure on a given day were divided by the measurements obtained upon filtered air exposure on the same day, which corresponds to the normalization to the filtered air exposure. The ratio obtained by F1 is equal to one when exhaust filtration has no influence on the biological response. Values bigger than one indicate that a certain biological response to filtered exhaust exposure is larger, values smaller than one that it is smaller than the response to unfiltered exhaust exposure (vice versa for GSH quantification). The results are listed in numerical form in **Supplementary Material Table S3**.

Statistical evaluation of the results was performed using the procedure described by Cumming and Finch (Cumming and Finch, 2005). All results are presented as average \pm standard deviation, *P*-values below 5% were considered statistically significant.

3. Results

3.1. Exhaust and particles

3.1.1. Exhaust composition

At the level of resolution available in this study, the use of a DPF did not influence the gaseous constituents of the exhaust. We detected a more than 99.999% decrease in the particle number concentration between the filtered and the unfiltered exhaust. Accordingly, also a large decrease in EC was detected. Note that the EC concentration in the filtered exhaust is below the detection limit of the photoelectric aerosol sensor and should therefore not be considered quantitative. The total active surface area could only be measured for the filtered exhaust because the diffusion charging sensor suffered from particle overload when unfiltered exhaust was used (all data listed in [Supporting Information Table S4](#) and [Supporting Information Figure S2](#)).

3.1.2. Particle deposition in the cell exposure chambers

For the determination of the number of particles deposited on TEM grids, a total number of 28 images were counted from 3 independent experiments (with two doses assessed). Exposures with unfiltered exhaust resulted in $1.7 \times 10^7 \pm 9.4 \times 10^6$ (2 h) and $7.4 \times 10^7 \pm 1.9 \times 10^7$ (6 h) DEPs deposited per cm^2 , the particles being homogeneously distributed. Instead of the expected three-fold, a 4.3-fold increase in the average number of deposited particles was observed when the exposure time was tripled. Given the large standard deviations (which are a consequence of the applied method) however, the results are in the expected range. When filtered exhaust was used, we could not detect any deposited particles ([Supporting Information Table S4](#)). Given the total surface area of the exposure chamber (2900 cm^2) the particle deposition rate was in the range of 0.5%.

3.2. Biological responses

As already mentioned in 2.11, the presence of primary cells in the used cell culture model increased the variation of the biological responses. This variation is an intrinsic property of the used biological system and could not be reduced by increasing the number of experimental repetitions, as exemplified for cell cultures exposed to unfiltered exhaust ([Supporting Information Fig. S4](#)). The standard deviation for up to 11 independent experiments is in the same range as for an n of 3. Particularly for the immune cells – the biologically most highly active component of the cell culture –, we considered this additional variation preferable over the exclusive use of cell lines, on one hand because responses of cell lines might not be representative for *in vivo* responses at all, on the other hand because this variation is the result of the variation among individuals in a human population and thereby even increases the value of the obtained results by describing the actual real-world situation.

3.2.1. Laser scanning microscopy

Laser scanning microscopy did not reveal any visible changes in cellular morphology, nor were any ruptures in the epithelial layer observed. This observation is independent on the exposure type or the exposure duration ([Fig. 1A](#)).

3.2.2. Cytotoxicity and pro-apoptotic response

No statistically significant changes in extracellular LDH activity were detected between filtered air and exhaust exposures ([Fig. 1B](#)) and an effect of the exposure duration was not detectable. The comparison between the unfiltered and filtered exhaust applying [formula F1](#) revealed a higher increase in extracellular LDH activity

(1.3 ± 0.3 (2 h) and 1.2 ± 0.2 (6 h)) for the filtered exhaust (not statistically significant) ([Fig. 4A](#)).

Results for *CASP7* and *FAS* expression are shown in [Figs. 1C](#) and [4A](#). Generally, real-time RT-PCR revealed very low expression levels for both genes (C_t higher than 30). These results should therefore not be considered quantitative and merely indicate the absence of pro-apoptotic stimulation.

3.2.3. Quantification of total reduced glutathione

Compared to the filtered air exposure, we detected statistically significant changes in the amount of total reduced GSH upon diesel exhaust exposure for filtered and unfiltered exhaust ([Fig. 2A](#)). Upon unfiltered exhaust exposure, we measured the 0.08 ± 0.1 -fold (2 h) and 0.07 ± 0.09 -fold (6 h) amount of reduced GSH compared to cell cultures that had been exposed to filtered air. For filtered exhaust exposure, the according values were 0.2 ± 0.07 (2 h) and 0.2 ± 0.08 (6 h). A dependency on the duration of the exposure was not detected. The DPF was found to have a statistically significant effect on GSH oxidation: Upon exposure to filtered exhaust, 2.7 ± 0.9 - (2 h) and 2.7 ± 1.0 (6 h)-fold higher levels of reduced GSH were detected than upon exposure to unfiltered exhaust ([Fig. 4B](#)).

3.2.4. Transcriptional activity of *HMOX1* and *SOD1*

The mRNA isolated from three of the six sets of cell cultures exposed to filtered exhaust contained large amounts of contaminations with high absorbance at 230 nm, which is why we decided not to further process these samples. As a consequence all data on gene expression for exposures to filtered exhaust are based on only three repetitions instead of six, which affects the reliability and the statistical significance of the results.

As shown in [Fig. 2B](#), real-time RT PCR revealed a 24 ± 15 - (2 h) and a 41 ± 30 -fold (6 h) increase of *HMOX1* expression upon unfiltered exhaust exposure compared to filtered air exposure. For filtered exhaust exposures, 23 ± 11 - (2 h) and 16 ± 9 -fold (6 h) expression levels were detected.

Only weak effects on *SOD1* expression were detected ([Fig. 2B](#)).

The Effects of the DPF on *HMOX1* and *SOD1* expression were weak (even though statistically significant for *SOD1* after 6 h exposure ([Fig. 4B](#))).

3.2.5. Transcriptional activity of *TNF* and *IL-8*

Only for *IL-8* (6 h) a significant increase of gene expression could be detected ([Fig. 3A](#)).

For *IL-8* expression, we detected a statistically significant influence of the DPF. Filtered exhaust resulted in an only 0.4 ± 0.1 - (2 h) and 0.3 ± 0.1 -fold (6 h) change in transcriptional activity compared to unfiltered exhaust ([Fig. 4C](#)).

3.2.6. Quantification of extracellular *TNF- α* and *IL-8*

For both cytokines, no significant difference between filtered air and exhaust exposure could be detected, independently on the exhaust type and the exposure duration ([Fig. 3B](#)). However, a significant, exposure duration independent influence of the DPF on both cytokines was observed (*TNF- α* : 0.6 ± 0.3 (2 h), 0.6 ± 0.2 (6 h), *IL-8*: 0.9 ± 0.08 (2 h), 0.8 ± 0.13) ([Fig. 4C](#)).

4. Discussion

As expected, the DPF reduced the particle number and the EC concentration in the exhaust almost completely and was more efficient for the smaller particle fraction ([Biswas et al., 2008; Mayer et al., 2010](#)). The absence of deposited particles in the cell exposure chambers upon exposure to filtered exhaust also confirms the high filtration efficiency of the DPF. It has to be emphasized however, that the formation of nucleation mode particles consisting of

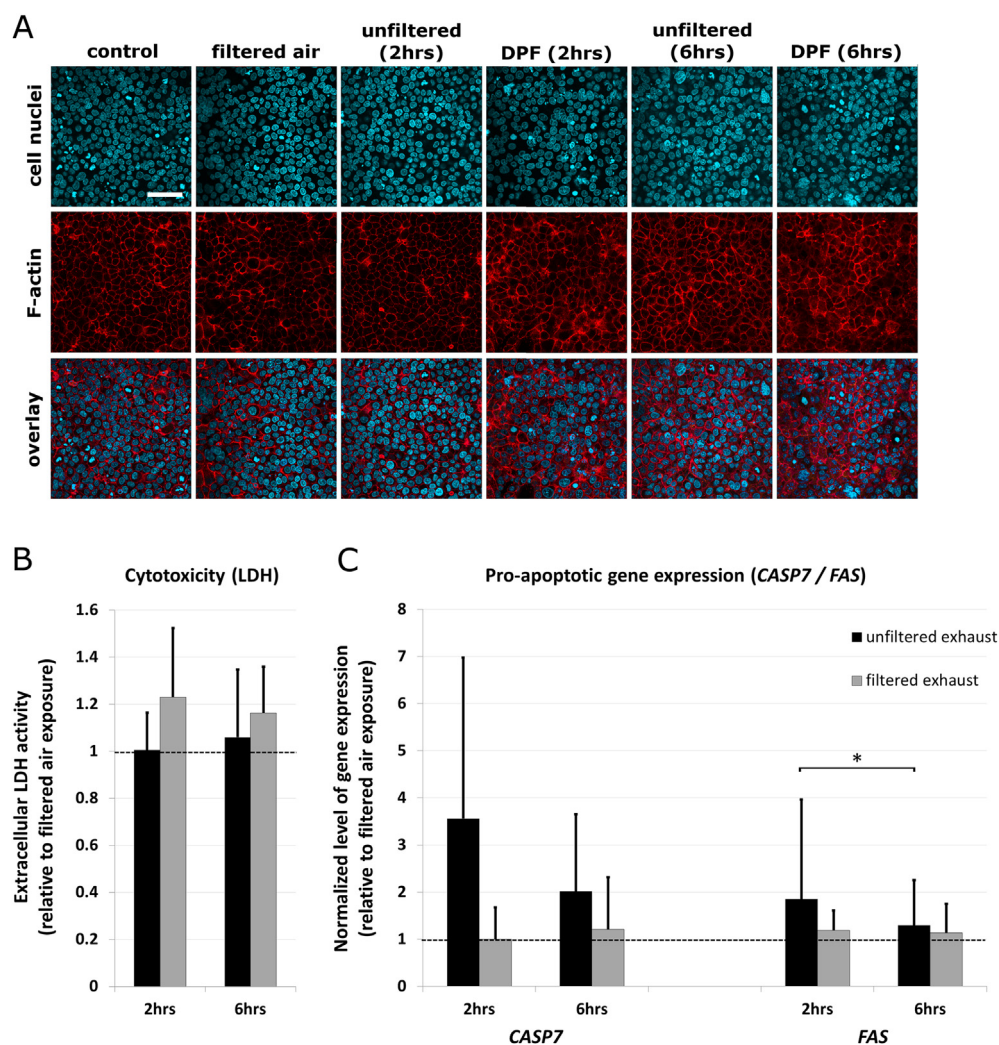


Fig. 1. Cytotoxicity, pro-apoptotic response and cellular morphology. A) Laser scanning microscopy, the size bar corresponds to 50 μm . B) Cytotoxicity (extracellular LDH activity), C) pro-apoptotic response (real-time RT-PCR on *CASP7* and *FAS* gene expression). All values are reported relative to the unfiltered air exposure (dotted line). Error bars indicate standard deviations. $N = 10$ (unfiltered exhaust) $N = 6$ (filtered exhaust, except for gene expression data, where $N = 3$). Asterisks indicate statistical significance ($p < 0.05$).

semivolatile organics (which cannot be detected by TEM) downstream the filter cannot fully be ruled out, even though such a fraction was not detected by the scanning mobility particle sizer. Changes in the gaseous composition of the exhaust were negligible

at the level of resolution available in this study. Literature on how non-catalyzed DPFs change the composition of the volatile hydrocarbon fraction could not be found, since the particle bound fraction of organic carbon is changed by non-catalyzed DPFs (Biswas

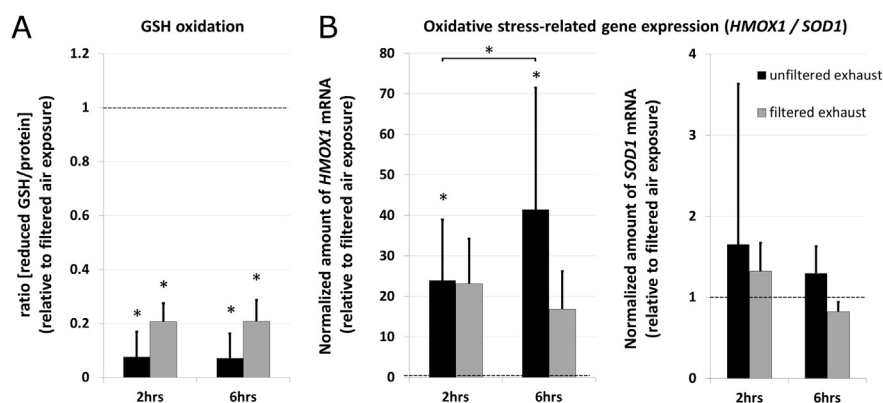


Fig. 2. Oxidative stress. A) levels of total reduced GSH, B) and C) cellular response to oxidative stress (real-time RT-PCR on *HMOX1* and *SOD1* gene expression). All values are reported relative to the unfiltered air exposure (dotted line). Error bars indicate standard deviations. $N = 10$ (unfiltered exhaust) $N = 6$ (filtered exhaust, except for gene expression data, where $N = 3$). Asterisks indicate statistical significance ($p < 0.05$).

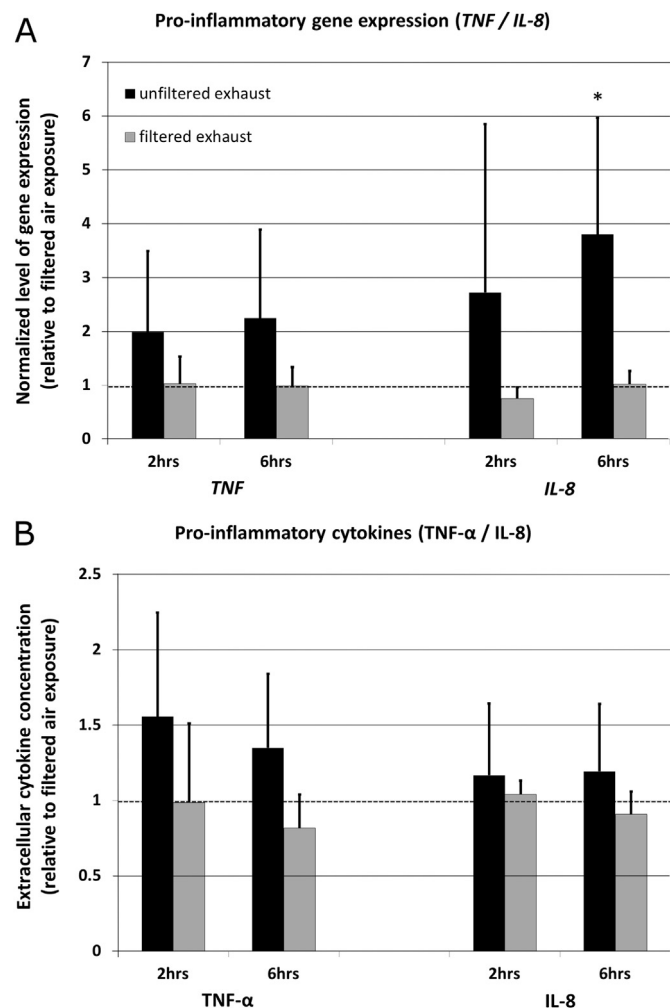


Fig. 3. Inflammatory response. A) And B) gene expression levels of *TNF* and *IL-8* (real-time RT-PCR), C) and D) extracellular *TNF-α* and *IL-8* concentrations (ELISA). All values are reported relative to the unfiltered air exposure (dotted line). Error bars indicate standard deviations. $N = 10$ (unfiltered exhaust) $N = 6$ (filtered exhaust, except for gene expression data, where $N = 3$). Asterisks indicate statistical significance ($p < 0.05$).

et al., 2009) however, we must assume that this is also true for the volatile fraction and it cannot be excluded that this influenced the assessed biological responses.

Based on the estimate that EC accounts for about 25–75% of the total particle mass (Biswas et al., 2009; Burtcher, 2005), we worked in the particle mass concentration range of 100–300 $\mu\text{g}/\text{m}^3$, which corresponds to what is only experienced at highly polluted sites (Weingartner et al., 1997). The rationale behind this choice was the comparability to other studies; in many *in vivo* and human exposure studies particle mass concentrations in the range of 100–300 $\mu\text{g}/\text{m}^3$ were used (Ghio et al., 2012a, 2012b; Lucking et al., 2011). Further, Finch (Finch et al., 2002) reported a roughly two-fold exhaust dilution to result in a particulate concentration 200 $\mu\text{g}/\text{m}^3$ and 25 ppm NO_2 , whereas Birger et al. (2011) reported a 250-fold dilution to result in 300 $\mu\text{g}/\text{m}^3$ and 7.4 ppm NO_2 . We worked with 100–300 $\mu\text{g}/\text{m}^3$ particles and 11 ppm NO_x . Hence, with higher exhaust dilution we would probably have underestimated the gas effects in relation to other studies. It should be emphasized here that no cytotoxicity, pro-apoptotic effects or morphological changes were detected upon exposure experiments (the increased average *CASP7* expression upon exposure to filtered

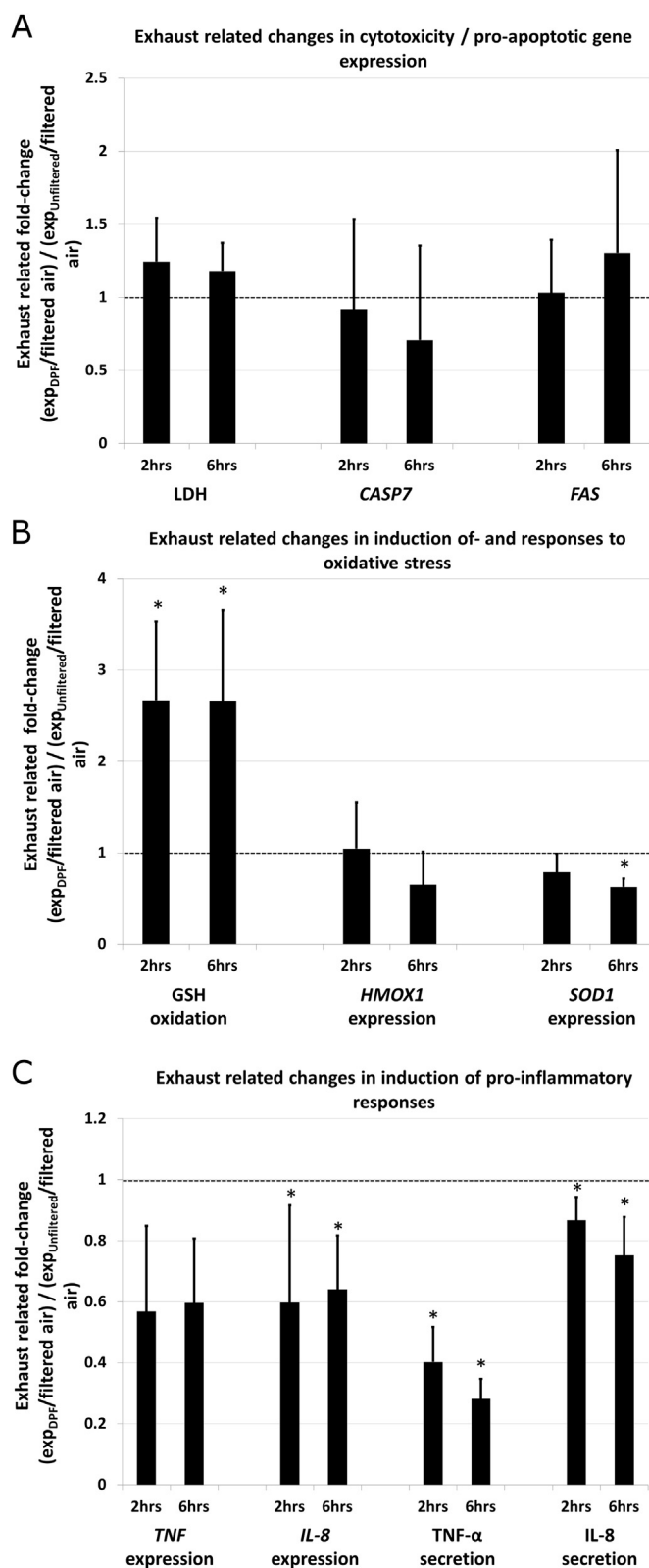


Fig. 4. Analysis of effects of filtered exhaust and unfiltered exhaust. A) Cytotoxicity and pro-apoptotic response. B) Induction of and response to oxidative stress. C) Inflammatory response. Calculations were done according to formula F1. The dotted line indicates the zero-effect level (where unfiltered exhaust = filtered exhaust), error bars indicate standard deviations. $N = 10$ (unfiltered exhaust) $N = 6$ (filtered exhaust, except for gene expression data, where $N = 3$). Asterisks indicate statistical significance ($p < 0.05$).

air/unfiltered exhaust for 6 h in the data set for unfiltered exhaust exposure results from a single strongly elevated value). Hence, the observed biological responses were the result of properly functioning regulatory networks of cells in a normal metabolic state and the used exhaust concentration did not cause an overload situation.

The inherent redox properties of DEPs are well described (Shinyashiki et al., 2009; Xiao et al., 2003) and since the DPF removed virtually all particles from the exhaust, we expected that exposure to filtered exhaust would result in considerably less GSH oxidation compared to unfiltered exhaust. The DPF indeed reduced the oxidative potential of the exhaust, but the effect was smaller than expected even though statistically significant. We therefore conclude that with respect to GSH oxidation, the chemistry of the gas phase (i.e. the presence of oxidizing species such as NO and NO₂, but also PAHs (Penning et al., 1999; Pryor et al., 1982)) is at least as important as the particles, a conclusion supported by the absence of a dose dependent GSH oxidation, which would be expected for particle related effects, but not for gas related ones.

The level of reduced GSH – or more precisely the ratio between reduced and oxidized GSH (GSH/GSSG) – is of great regulatory importance. A decrease of GSH/GSSG and hence the loss of reducing conditions in a cell influences a variety of signaling processes, which is thought to at least partly rely on oxidative modifications and thereby activation or inactivation of regulatory proteins (Biswas et al., 2009; Rahman, 2003). Such redox-sensitive signaling allows a cell to sense the presence and the intensity of oxidative stress and to mount the appropriate responses, which include anti-oxidant defence and – at higher levels of oxidative stress – pro-inflammatory reactions (Li et al., 2008; Reuter et al., 2010; Xiao et al., 2003). On the basis of such a network of responses, our data indicate that irrespective of the exhaust type used for exposure, anti-oxidant responses such as *HMOX1* expression are activated and that the difference between the levels of reduced GSH, i.e. in the severity of the oxidative stress are not biologically relevant in this regard.

Unfiltered exhaust clearly induced the production and the release of TNF- α and IL-8, whereas filtered exhaust did not. Whether the difference in the induction of pro-inflammatory responses is the result of the lower oxidative potential of the filtered exhaust or independent on the redox state of the cell cultures can only be hypothesized, even though this is exactly what would be expected based on the above mentioned oxidative stress-responsive signalling mechanisms. Reduced GSH is however, strongly depleted upon exposure to both filtered and unfiltered exhaust and relative to the homeostatic GSH levels (filtered air exposure), the difference between the two appears to be too small to be biologically relevant. Moreover, a certain dose-dependency for unfiltered exhaust-induced IL-8 gene expression was observed. This is difficult to explain by the levels of reduced GSH which were dose independent, but rather suggests a role of the particle load on the cell cultures.

Even though our results are in general agreement with the literature (Gerlofs-Nijland et al., 2013; Schwarze et al., 2013), one should keep in mind that a direct comparison is in most cases not possible, since different cell types, exposure conditions and -doses have been used (Holder et al., 2008). Particularly, in our experiments, complete exhaust was used for exposures of cell cultures cultivated at the air-liquid interface whereas most other studies have been performed by exposing suspended cell cultures to particle solutions, which makes dose-comparison basically impossible. Even if the applied doses are comparable, exposure in suspension tends to under-estimate exhaust toxicity (Lichtveld et al., 2012).

It should further be kept in mind that the results presented here apply only for the tested system, which refers to the used test-engine, the DPF, the fuel, the applied (constant) driving cycle, and

the absence of filter regeneration and secondary organic aerosol formation (exhaust ageing), all of which are known to influence the emission in terms of composition and/or quantity (Bensaid et al., 2011; Maricq, 2007; Samy and Zielinska, 2010). The constant driving cycle and the absence of exhaust ageing and filter regeneration represent simplified, non-real-world scenario. This was chosen deliberately however, in order to work under highly standardized conditions.

In summary, our results allow differentiating the contributions of the particulate and the gaseous fraction to the overall exhaust toxicity. We could show that the use of a non-catalyzed DPF – even though lowering the oxidative potential of diesel exhaust – is not sufficient to eliminate the induction of oxidative stress, but efficiently inhibits the induction of inflammatory responses upon exhaust exposure *in vitro*.

We conclude that i) removal of the particulate fraction of diesel exhaust is a crucial step towards exhaust-detoxification, since it is well documented that acute local inflammation is the starting point for many of the adverse acute and long-term consequences of diesel exhaust exposure (Li et al., 2008; Schins and Knaapen, 2007). ii) effects of gaseous exhaust components cannot be neglected, since they are sufficient to induce oxidative stress and – based on what is known about the connection between oxidative stress and inflammation (Donaldson et al., 2005; Reuter et al., 2010; Xiao et al., 2003) – by doing so are likely to induce pro-inflammatory responses over long term. What is now needed is research on how the effect of a DPF on exhaust toxicity can be improved – or corrupted – by the addition of catalytically active systems. In future studies, it should therefore be investigated how the results presented here change when e.g. catalytically coated DPFs or DPFs in combination with diesel oxidation catalysts are used.

Conflict of interest

Andreas Mayer is the owner and general manager of “TTM Andreas Mayer”, Switzerland, an emission consulting company. As all the other authors however, he declares to have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atmosenv.2013.08.029>.

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