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Assessment of lung cell toxicity of various gasoline engine exhausts using a versatile *in vitro* exposure system☆

Christoph Bisig [a], Pierre Comte [b], Martin Güdel [b], Jan Czerwinski [b], Andreas Mayer [c], Loretta Müller [d], Alke Petri-Fink [a], Barbara Rothen-Rutishauser [a, *]

[a] Adolphe Merkle Institute, University of Fribourg, Switzerland
[b] Laboratory for Exhaust Emission Control, Bern University of Applied Sciences, Biel-Bienne, Switzerland
[c] Technik Thermische Maschinen, Switzerland
[d] University Children's Hospital Basel, Switzerland

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ABSTRACT

Adverse effect studies of gasoline exhaust are scarce, even though gasoline direct injection (GDI) vehicles can emit a high number of particles.

The aim of this study was to conduct an *in vitro* hazard assessment of different GDI exhausts using two different cell culture models mimicking the human airway. In addition to gasoline particle filters (GPF), the effects of two lubrication oils with low and high ash content were assessed, since it is known that oils are important contributors to exhaust emissions.

Complete exhausts from two gasoline driven cars (GDI1 and GDI2) were applied for 6 h (acute exposure) to a multi-cellular human lung model (16HBE14o-cell line, macrophages, and dendritic cells) and a primary human airway model (MucilAir™). GDI1 vehicle was driven unfiltered and filtered with an uncoated and a coated GPF. GDI2 vehicle was driven under four settings with different fuels: normal unleaded gasoline, 2% high and low ash oil in gasoline, and 2% high ash oil in gasoline with a GPF. GDI1 unfiltered was also used for a repeated exposure (3 times 6 h) to assess possible adverse effects.

After 6 h exposure, no genes or proteins for oxidative stress or pro-inflammation were upregulated compared to the filtered air control in both cell systems, neither in GDI1 with GPFs nor in GDI2 with the different fuels. However, the repeated exposure led to a significant increase in *HMOX1* and *TNFa* gene expression in the multi-cellular model, showing the responsiveness of the system towards gasoline engine exhaust upon prolonged exposure.

The reduction of particles by GPFs is significant and no adverse effects were observed *in vitro* during a short-term exposure. On the other hand, more data comparing different lubrication oils and their possible adverse effects are needed. Future experiments also should, as shown here, focus on repeated exposures.

1. Introduction

It has been shown by many studies that exposure to particulate matter smaller than 2.5 μm ($PM_{2.5}$) correlates with cardiovascular and pulmonary diseases as well as cancer (Brunekreef and Holgate, 2002; Pope et al., 2002). A recent meta-analysis of 110 peer-reviewed time series also showed that a 10 μg/cm^3 $PM_{2.5}$ increment leads to a 1.04% increased risk of death (Atkinson et al., 2014). The pathway by which air pollution affects human health is via inhalation of particles and gases inducing local cellular reactions in the lung tissue, such as oxidative stress followed by (pro-)inflammation, DNA damage, and direct cytotoxic effects. Ambient

Abbreviations: ALI, air-liquid interface; CO, carbon monoxide; CO_2, carbon dioxide; cLSM, confocal laser scanning microscope; CVS, constant volume sampler; ELISA, enzyme-linked immunosorbent assay; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GDI, gasoline direct injection; GPF, gasoline particle filter; HC, hydrocarbons; HMOX1, heme oxygenase 1; CXCL8, interleukin 8; LDH, lactate dehydrogenase; MDDC, monocyte derived dendritic cells; MDM, monocyte derived macrophages; NOx, nitric oxides; NQO1, NAD(P)H dehydrogenase [quinone] 1; PAH, polyaromatic hydrocarbons; PBS, phosphate-buffered saline; PN, particle number; PM, particulate matter; SOD2, superoxide dismutase 2; TNFa, tumor necrosis factor alpha; WLTC, Worldwide-harmonized Light vehicles Test Cycle.

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* Corresponding author. Chemin des Verdiers 4, 1700, Fribourg. Switzerland.
E-mail address: barbara.rothen@unifr.ch (B. Rothen-Rutishauser).

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particles or secondary mediators can also reach the vascular system and other organs, potentially resulting in ischemic heart disease or stroke (reviewed in Risom et al., 2005; Steiner et al., 2016; Stone et al., 2017).

Major contributors to air pollution are traffic related combustion processes, such as diesel and gasoline vehicles. While adverse effects of diesel exhausts have been widely studied (e.g Hashimoto et al., 2001; Steiner et al., 2013; Zarcone et al., 2016), effects from gasoline vehicles are less known. This lack of toxicological data does not reflect the current trends for the use of gasoline vehicles in comparison to diesel cars. In addition, the modern gasoline direct injection (GDI) technology release significantly higher particle number (PN) emissions compared to older gasoline vehicles or modern diesel vehicles equipped with a particle filter (Zhang and McMahon, 2012; Platt et al., 2017). It was also reported that GDI vehicles need a gasoline particle filter (GPF) to comply with the current Euro6 PN-legislation in the future (Czerwinski et al., 2017).

To the best of our knowledge, only one other group investigated *in vitro* pulmonary effects of a GDI vehicle (Maikawa et al., 2016), but with only few biological parameters. More data is needed using a realistic exposure system, that includes freshly produced exhaust and cells at the air-liquid interface (ALI). Additionally experiments with GPF's are needed, as filtration alone has not always been proven to be sufficient for exhaust detoxification (Holder et al., 2007; McDonald et al., 2007; Steiner et al., 2014).

Vehicle exhaust emissions are not only influenced by the engine type, but also by the fuel composition (e.g. ethanol or butanol supplement, octane number), after-treatment system (exhaust gas recirculation, three-way catalyst, and particle filter), ambient conditions, and lubrication oil. It has been shown that lubrication oil significantly contributes to the PN emissions of diesel (Buchholz et al., 2003; Brandenberger et al., 2005) and gasoline (Sonntag et al., 2012) engines, and polyaromatic hydrocarbons (PAHs) have also been shown to be derived (at least partly) from unburnt lubrication oil (Geller et al., 2006). As vehicle mileage increases, the usage of lubrication increases due to engine wear (gasket, valve, and piston condition) (Pedersen et al., 1980; Robert et al., 2007), however, driving style and oil viscosity also influence oil consumption. Despite the known influence of lubrication oil on engine emissions, no regulation is in place for the composition of lubrication oils. Importantly, no study could be found that simulated consumption of different lubrication oils and investigated the *in vivo* or *in vitro* toxicology of such exhausts (or extracts).

We have recently established an *in vitro* exposure system (Muller et al., 2010); the heated system contains two exposure chambers holding up to four 6-well cell culture plates each. The system is versatile and can be directly connected to any engine; this far it has been used with scooter (Müller et al., 2011), diesel (Steiner et al., 2012; Steiner et al., 2013a; Steiner et al., 2013b; Steiner et al., 2014), and gasoline (Bisig et al., 2015; Bisig et al., 2016) vehicles, resulting in reproducible and sensitive data that allowed differentiation between the particle and gaseous fractions in exhaust emissions. Using this set-up we have shown that exhaust emissions, *i.e.* 40% of the particulate fraction, is deposited on inserts which are placed in the 6-well plates, indicating that the exhaust can interact with the cellular fraction (Muller et al., 2010).

Herein we have taken further advantage of the versatility of our exposure system to test the influence of various parameters (Fig. 1). Two different GDI vehicles were used in single acute exposures of 6 h, vehicle GDI1 was driven unfiltered (GDI1 reference) as well as with two different filters (uncoated and coated GPF). A coated GPF was included because it can also remove volatile organic compounds. Vehicle GDI2 was driven unfiltered with normal gasoline (GDI2 reference) and with modified gasoline, *i.e.* two different lube-oils (high ash and low ash content) were added in 2% volume, the one with high ash was additionally driven with a filter (high ash GPF). A GPF was attached because it was hypothesized that the high ash lube-oil would have the highest negative impact on both exhaust emissions and lung-cell cultures, and a filter would diminish some of the effects. Since humans can be constantly exposed to exhaust emissions over a longer period of time, the GDI1 reference was driven three times for 6 h and possible adverse effects of this repeated exposure were assessed in the lung cell cultures to mimic a prolonged exposure.

Lung cell models mimicking the respiratory airway tract were used. The first lung model, the multi-cellular lung model (Rothen-Rutishauser et al., 2005), consisting of a bronchial cell-line supplemented with primary macrophages and dendritic cells, was used for previous hazard identification studies and clearly demonstrated a significant elevation of oxidative stress and pro-inflammatory stress markers upon exposure to unfiltered diesel exhaust, allowing a comparison of the results (Steiner et al., 2012; Steiner et al., 2013; Steiner et al., 2014; Bisig et al., 2015; Bisig et al., 2016). The second model, the MucilAir™ system consists of primary human bronchial epithelial cells, and includes mucus-producing as well as ciliated cells (Huang et al., 2013), two important features in the defense against particulates. The two models complement each other, as one includes immune cells while the other can produce mucus and has functional cilia. It was hypothesized that they will react differently to gasoline exhaust, giving a better overall understanding of the *in vitro* effects of gasoline exhausts.

The major cellular responses induced by traffic-derived air pollutants are oxidative stress and (pro-)inflammation (Xiao et al., 2003; Risom et al., 2005). Therefore, gene expression analysis was performed with genes related to these two endpoints and selected based on their upregulation in other studies with diesel exhaust, diesel exhaust particles only, or gasoline exhaust (e.g Gong et al., 2007; Huang et al., 2011; Wittkopp et al., 2016).

2. Materials and methods

2.1. Test vehicles, exposure system and protocol

Gasoline passenger cars (GDI technology) were driven on a chassis dynamometer following the Worldwide harmonized Light vehicles Test Cycle (WLTC) protocol for 6 h (ten cycles), which included one cold start.

A modern flex-fuel car (Euro5b, 2012, mileage during exposure 15271–21608 and 27693–28600 km), herein defined as GDI1, was previously tested with different ethanol-gasoline fuel blends (Bisig et al., 2016). The GDI1 car was driven unfiltered (GDI1 reference) as well as with two different filters on conventional unleaded gasoline. The uncoated GPF (as used in Bisig et al., 2015) had a pore size of 19 μm and 50% porosity while the coated GPF had a smaller pore size (14 μm) and higher porosity (55%). Both GPFs (cordierite, 200 cells per square inch) were installed approximately 60 cm downstream from the original three-way catalyst.

As the GDI1 vehicle was not available during the lubrication-oil experiments and also to compare different GDI technologies, a second GDI car was used. The second car (GDI2, Euro6b, 2014, mileage during exposure 27912–31345 km) was driven unfiltered on normal gasoline (GDI2 reference) as well as with two lubrication-oil-enriched fuels to simulate high oil consumption. The oils with equal viscosity were each mixed with gasoline (2% V/V). Ash and metal content differed in the two used oils, high-ash (≤1.2%) and low-ash oils (≤0.5%) were used. It was hypothesized that the high-ash oil would have an impact on both emission and biological endpoints, therefore a freshly thermally-regenerated, uncoated GPF (as used in Steiner et al., 2013) was mounted at the