Markers of lipid oxidation and inflammation in bronchial cells exposed to complete gasoline emissions and their organic extracts

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ABSTRACT

Road traffic emissions consist of gaseous components, particles of various sizes, and chemical compounds that are bound to them. Exposure to vehicle emissions is implicated in the etiology of inflammatory respiratory disorders. We investigated the inflammation-related markers in human bronchial epithelial cells (BEAS-2B) and a 3D model of the human airways (MucilAir™), after exposure to complete emissions and extractable organic matter (EOM) from particles generated by ordinary gasoline (E5), and a gasoline-ethanol blend (E20; ethanol content 20% v/v). The production of 22 lipid oxidation products (derivatives of linoleic and arachidonic acid, AA) and 45 inflammatory molecules (cytokines, chemokines, growth factors) was assessed after days 1 and 5 of exposure, using LC-MS/MS and a multiplex immunoassay, respectively. The response observed in MucilAir™ exposed to E5 gasoline emissions, characterized by elevated levels of pro-inflammatory AA metabolites (prostaglandins) and inflammatory markers, was the most pronounced. E20 EOM exposure was associated with increased levels of AA metabolites with anti-inflammatory effects in this cell model. The exposure of BEAS-2B cells to complete emissions reduced lipid oxidation, while E20 EOM tended to increase concentrations of AA metabolite and chemokine production; the impacts on other inflammatory markers were limited. In summary, complete E5 emission exposure of MucilAir™ induces the processes associated with the pro-inflammatory response. This observation highlights the potential negative health impacts of ordinary gasoline, while the effects of alternative fuel are relatively weak.

1. Introduction

Road traffic significantly contributes to air pollution and negatively impacts human health. Diesel engine emissions have been classified as carcinogenic to humans (IARC Group 1), and the gasoline engine exhaust is a possible human carcinogen (IARC Group 2 B) (IARC, 2013). Given the current pressure to reduce the number of diesel-powered vehicles and give preference to gasoline-powered vehicles, the investigation of the health effects of gasoline emissions, including the exhaust from various blends of gasoline with ethanol, has become a priority. In general, the negative health impacts of air pollutants stem from a combination of the effects of particles, especially fine particles [aerodynamic diameter < 2.5 μm (PM2.5)], chemical compounds adsorbed to them and gaseous components (Almetwally et al., 2020). On the molecular level, the adverse effects of air pollutants are linked with the damage of cellular macromolecules leading to the formation of DNA or protein adducts, along with nucleic acid strand breaks and rearrangements that may lead to mutations and the initiation of carcinogenesis. Oxidative stress was identified as one of the processes responsible for the damage of macromolecules. This process, initiated by reactive oxygen species (ROS), impacts nucleic acids, lipids, and proteins. Products of lipid peroxidation are of particular concern, as they are highly reactive, may attack other macromolecules, and propagate oxidative damage (Su et al., 2019). Furthermore, unlike DNA, there are no mechanisms that would repair oxidized lipid molecules. The particulate components of air pollutants are linked with the induction of an inflammatory response (Arias-Pérez et al., 2020) that is implicated in many diseases, such as pulmonary, cardiovascular and neurological disorders, as well as cancer. This response, characterized by the production of cytokines, chemokines and other inflammatory molecules, and accompanied by the generation of ROS, is elicited not only by the cells of the immune system, but also by the airway epithelial cells that are in the first line of contact with polluted air (Takizawa et al., 2000). Inflammatory processes are closely linked with lipid oxidation, in which polyunsaturated fatty acids [PUFA; particularly linoleic acid and arachidonic acid (AA)] serve as precursors for the synthesis of potent pro-inflammatory mediators, such as prostaglandins (PGs), leukotrienes (LXs), hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs) (Araújo et al., 2018; Innes and Calder, 2018). AA is generated from linoleic acid by a series of enzymatic reactions that involve desaturases and an elongase. AA is cleaved from cell membranes by the activity of phospholipase A2, and further metabolized by cyclooxygenases, lipoxygenases and cytochrome P450 enzymes to HETEs, PGs, LXs and thromboxanes, that regulate inflammatory processes (Innes and Calder, 2018). Linoleic acid, an inflammatory mediator, can be converted by the activity of lipoxygenases to HODEs, molecules that are also involved in inflammation (Innes and Calder, 2018). On the molecular level, a link between inflammatory and lipid peroxidation processes is established by ROS generated during oxidative stress that activates NF-κB. This transcription factor regulates the expression of numerous genes, including those encoding cyclooxygenases, cytokines or growth factors (Hoesel and Schmid, 2013). Among other cell types, lipid peroxidation products are generated by activated immune cells at the site of local injury, and act as promoters or suppressors of inflammatory processes.

Biofuels have been introduced for use in road vehicles with the aim to reduce fossil fuel consumption, address global climate change and improve air quality, particularly in large cities. Biodiesel and ethanol-based gasoline fuels represent the most commonly used alternative fuels. Questions have arisen concerning the chemical composition and the toxicity of biofuel emissions, due to their increasing production and the combustion in passenger vehicles. The current data suggest that an increasing content of ethanol in gasoline results in the increased emissions of its oxidation products, including acetaldehyde, a possible human carcinogen (IARC, 1999), and decreased levels of NOx and CO. There is no consensus yet on the production of total hydrocarbons and ozone formation after burning this fuel (Wallington et al., 2016). The impacts of biodiesel burning on human health have been investigated in several studies (Godri Pollitt et al., 2019), suggesting a biological response comparable to standard diesel. For gasoline blends with ethanol, studies are limited to *in vitro* investigations in various model systems (Agarwal et al., 2020; da Silva et al., 2019; Hakkarainen et al., 2020; Roth et al., 2017; Yang et al., 2019), and the replication of their results in humans is lacking.

Although the evaluation of toxicity in humans (volunteers or entire populations) represents the ultimate confirmation of the potential negative effects of tested compounds, such an approach may not be practical/ethical in certain scenarios. Thus, to overcome these obstacles, various model systems are used in toxicology. The selection of a suitable model is paramount to obtain results that could be extrapolated to human organisms. In recent years, 3D tissue cultures grown at the air-liquid interface (ALI) have become the new standard in the toxicity testing of air pollutants (McKim, 2015). The ALI tissue cultures allow assessment of the effects of complete emissions, i.e. gaseous and particulate components, including compounds adsorbed onto the surface of particles. This approach overcomes the limitations of many studies that have tested the effects of either particles alone (collected in filters and subsequently reconstituted in a suitable solvent), or the extracts of compounds adsorbed to the particles, thus omitting the combined impact of these components on the organism. 3D cultures exposed at the ALI thus represent a realistic model for the toxicity testing for relevant target organs, that help to replace the still commonly used submerged monolayer cell cultures (Upadhyay and Palmberg, 2018).

In this study, we focused on the comparison of biological impacts induced by ordinary gasoline (denoted E5) and an ethanol-gasoline blend (20%, v/v; denoted E20), in two pulmonary models: a monolayer culture of human bronchial epithelial cells (BEAS-2B) and a 3D lung tissue model (MucilAir™). The exposure was either conducted at the ALI using complete engine emissions, or in submerged cultures exposed to organic extracts from particulate matter obtained from complete E5 and E20 emissions. We concentrated specifically on the analysis of a panel of 45 immunomodulatory molecules and 22 lipid oxidation products, derivatives of arachidonic and linoleic acids.

2. Materials and methods

2.1. Cell cultures

This study is a follow-up of our previously reported data in which we investigated the toxic response in BEAS-2B cells and MucilAir™ exposed to complete E5 and E20 emissions (Cervena et al., 2020; Rossner et al., 2019a). Therefore, detailed information on cell cultures and exposure conditions to complete emissions has already been published. Briefly, BEAS-2B cells obtained from American Tissue Culture Collection (ATCC; CRL-9609TM, ATCC®, Manassas, VA, USA) were grown in a serum-free medium (BEGMTM kit CC-3170; Lonza, Basel, Switzerland), while for MucilAir™ tissues (Epithelix Sàrl, Geneva, Switzerland), a culture medium provided by the manufacturer was used. Both cell models were kept at standard cultivation conditions (37 °C, 5% CO_2, relative humidity > 90%) in 24-well Transwell® cell inserts (Sigma-Aldrich, St Louis, MO, USA).

2.2. Exposure to complete emissions and extractable organic matter from particulate matter (EOM)

2.2.1. Complete emissions

Exposure followed a previously published protocol (Rossner et al., 2019a; Vojtisek-Lom et al., 2019). The experiments were performed using a Euro 5 direct injection spark ignition engine that ran on commonly available gasoline (E5; BA-95 N, Čepro, 4.9% ethanol, 0.3% ETBE), or a mixture of ordinary gasoline with ethanol to a final ethanol content of 20% (v/v) (E20). Raw exhaust was diluted with filtered air (10:1) and used simultaneously for real-time exposure of cell models in

an exposure box (Vojtisek-Lom et al., 2019), and for the collection of particulate matter onto fluorocarbon-coated glass filters (PallFlex, Pall, Portsmouth, UK) for the later preparation of organic extracts. The dilution was selected based on our pilot experiments in which BEAS-2B cells were stable in the exposure chamber for an extended period of time without visible changes (Vojtisek-Lom et al., 2019). In addition, this dilution is used as the most widely used approach in exhaust toxicity studies. On the filters, all the particles were collected, without size segregation, as the vast majority of particle mass was expected to be within 1 μm range (PM1). The exposure conducted for 1 and 5 days, respectively, followed the scheme previously described in detail (Rossner et al., 2019a; Vojtisek-Lom et al., 2019). The scheme, designed to mimic realistic human exposure to the engine exhaust, included cold starts and two runs of World Harmonized Light Vehicle Test Cycle (WLTC) in each exposure day. The control samples were exposed to filtered ambient air.

2.2.2. Extractable organic matter from particulate matter

Particles collected onto filters were extracted with 60 ml of dichloromethane and 3 ml of cyclohexane for 3 h. Extractable organic matter (EOM) was used for a detailed quantitative chemical analysis of PAHs, and their derivatives performed by HPLC with fluorimetric detection. The analysis included the detection of a total of 54 polycyclic aromatic hydrocarbons (PAHs), including IARC Group 1, 2 A and 2 B PAHs (carcinogenic PAHs, c-PAHs) and US Environmental protection Agency (US-EPA) priority PAHs. An overview of particulate matter mass collected onto the filters and deposited on inserts, PAHs content in EOM and doses of PAHs to which the cells in the individual inserts were exposed, is presented in Supplementary File 1. For cell exposure *in vitro*, samples of EOM were evaporated under a stream of nitrogen, and the residue dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 μg EOM/μl. The exposure scheme, adjusted to be comparable with the time schedule of exposure to complete emissions, included 1 h incubation in a culture medium, 1 h treatment with 15 μl of individual EOMs [a final concentration of 5 μg/ml (75 ng/insert) selected as non-cytotoxic based on pilot experiments with concentrations ranging from 0.1 to 10 μg/ml; data not shown], 2 h incubation in a culture medium and a final 1 h incubation step with 15 μl of EOM. For the 1 day exposures, the cells/media were collected after the treatment and stored at −80 °C for further analyses. For the 5 day exposures, the medium was replaced with a fresh one without EOM, and the cells were grown overnight. The following day, the exposure commenced as described above. EOMs from E5 and E20 used for the exposure experiments were prepared from a comparable amount of PM, although the concentration of PAHs differed. Doses of PAHs in EOM exposure experiments were about 15-fold and 24-fold higher than estimated doses of PAHs in PM deposited on the inserts for E5 and E20 treatment, respectively (Supplementary File 1). The dose of particles deposited on the inserts was estimated based on the gravimetric analysis of PM, the mean mass concentration of particles and particle concentrations, considering particle losses and a deposition rate of 2% as reported in our previous study (Cervena et al., 2020).

For the analysis of the parameters further described, a tissue culture medium collected after 1 day (T1) and 5 days (T5) of exposure was used. For each time point, samples from exposed and control cell cultures were obtained.

2.3. Detection of arachidonic acid and lipid oxidation products

The analysis was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), as previously described (Rynning et al., 2018). Briefly, the analyzed compounds were extracted from 4.5 ml of basal tissue culture medium, using solid phase extraction on SELECT HLB SPE cartridges (Supelco, Prague, Czech Republic). After the extraction, the samples were re-dissolved in methanol (60 μl) and injected into the HPLC column. For the LC-MS/MS, an Agilent 1200

chromatographic system (Agilent Technologies, Waldbronn, Germany) and a triple quadrupole mass spectrometer Agilent 6410 Triple Quad LC/MS (Agilent Technologies, Santa Clara) with an electrospray interface (ESI), were used. The mass spectrometer was operated in the negative ion mode. Selected ion monitoring at *m/z* 303.2 was used for the quantification of AA, and multiple-reaction monitoring for other analytes. Due to the sensitivity limits of the analytical method and low concentrations of analytes in the culture medium, basal media from treated biological triplicates were pooled for further analysis by LC-MS/MS.

2.4. Analysis of cytokines, chemokines, and growth factors

The production of selected cytokines, chemokines and growth factors into basal tissue culture media was assessed using the Human Cytokine/Chemokine/Growth Factor 45-Plex ProcartaPlex Panel (Thermo Fisher Scientific, Waltham, MA, USA; cat. no. EPX450-12171-901). The analysis was performed in a service laboratory of Thermo Fisher Scientific (Bender MedSystems GmbH, Vienna, Austria). The tissue culture medium samples from three independent biological replicates were pooled and 150 μl of the samples shipped on dry ice to the service laboratory. The detection performed in technical duplicates was done on a Luminex™ system; the data was analyzed and both the raw and processed fluorescence values were e-mailed to the Institute of Experimental Medicine for further evaluation.

2.5. Processing and visualization of the data

In this study, a comparison of 22 lipid peroxidation products and 45 immune response-related molecules assessed at different experimental conditions (two cell models, two exposure approaches) was carried out. In order to standardize data presentation and allow the comparison between exposure scenarios, we calculated relative values of analyzed markers in the exposed samples in relation to the controls separately for the individual time points. Specifically, the control samples at T1 served as a baseline for calculation of the relative increase/decrease of levels of the analyzed parameters in the exposed cells at this exposure time. An analogical approach was used for the samples obtained at T5. The relative changes greater than 1.5 or lower than 0.66 were considered biologically significant, and were presented in figures in red or green color (for increase or decrease of the analyzed parameter in the exposed samples, respectively). The raw data used for the above-mentioned standardization are available in Supplementary Files 3 and 4. Due to the specific requirements of analytical methods, the pooled biological triplicates were analyzed, and the standard statistical analyses could not be performed. Thus, the term "significant" used in this study refers to the biological significance as defined above. For the multiplex immunoassay, the differences between technical replicates of the exposed and control samples were further evaluated using Student's t-test; p-values ≤ 0.05 were considered significant. The results identified as biologically significant agreed with those detected as statistically significant by the *t*-test. Additionally, to further confirm validity of the data obtained from the pooled samples analyses, a general experimental variation of the MucilAir™ and BEAS-2B systems was evaluated using the results from other parameter measurements, including lactate dehydrogenase activity and transepithelial electrical resistance [(Cervena et al., 2020; Rossner et al., 2019a), unpublished data]. The average coefficients of variation of these data for individual exposure conditions and model systems were as follows: MucilAir™ exposed to complete emissions: 10.98%; BEAS-2B exposed to complete emissions:10.36%; MucilAir™ exposed to EOMs: 8.04%; BEAS-2B exposed to EOMs/: 7.82%. These results confirm low experimental variability of both model systems indicating that the pooled sample evaluation was a valid approach for lipid oxidation and inflammatory marker detection.

3. Results

3.1. Arachidonic acid and lipid oxidation products

Arachidonic acid (AA), eicosanoids and derivatives of linoleic acid were included among the PUFA and lipid oxidation products analyzed in our study. Therefore, we present the data for 16 molecules whose levels were detectable. The concentration of six compounds (13, 14-dihydro-15-keto-PGD$_2$, 15-keto-PGE$_2$, 6-keto-PGF$_{1a}$, PGJ$_2$, LXA$_4$, 20-HETE) were below the detection limits of the analytical methods for any combination

of the cell model/exposure condition (exposure time, tested compound), and are no longer discussed in this work (Supplementary File 2).

Complete emissions from both fuels elicited a relatively weak response in MucilAir™, while in BEAS-2B cells the response was generally more pronounced. In MucilAir™, E5 emissions exposure tended to increase the levels of lipid oxidation products, particularly PGE$_2$, PGF$_{2\alpha}$ and PGF$_{2\beta}$, while the exposure to E20 emissions was rather linked with decreased levels of the analyzed markers. In BEAS-2B cells, E5 emissions exposure was associated with elevated concentrations of several HETE derivatives and the decreased production of some
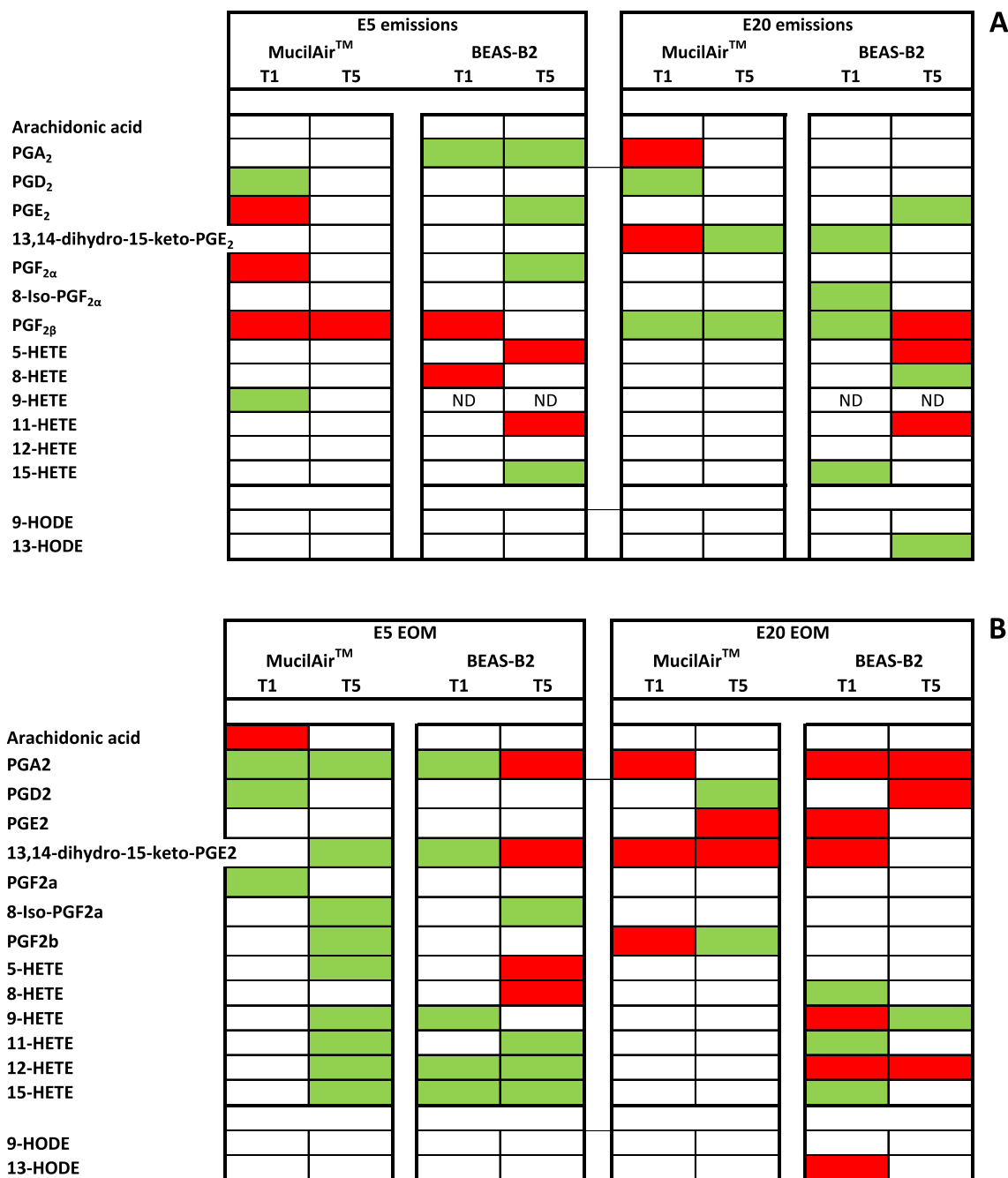
Fig. 1. 1A. Lipid oxidation products detected in culture media collected from MucilAir™ and BEAS-2B cells exposed to complete emissions from E5 and E20 fuels at time points T1 and T5. Red and green colors denote increased (>1.5) and decreased (<0.66) levels of the respective marker, when compared with the control at the individual time point. ND, not detectable. **1B.** Lipid oxidation products detected in a culture media collected from MucilAir™ and BEAS-2B cells, exposed to EOMs from complete emissions from E5 and E20 fuels at time points T1 and T5. Both EOMs were prepared from the comparable amount of PM. Red and green colors denote increased (>1.5) and decreased (<0.66) levels of the respective marker, when compared with the control at the individual time point. ND, not detectable. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

prostaglandins (PGA$_2$, PGE$_2$, PGF$_{2\alpha}$) and 15-HETE. For E20 emissions; the reduction of concentrations of lipid oxidation products was mostly found for this cell line. Levels of PGF$_{2\beta}$ were affected by most of the investigated conditions; concentrations of 13, 14-dihydro-15-keto-PGE$_2$ changed as a result of exposure to E20, but not E5 emissions. For some molecules, including e.g. arachidonic acid, no significant changes resulting from the exposure to complete emissions were detected (Fig. 1A; Supplementary File 3).

A

2A. E5 emissions / E20 emissions (red = increased >1.5; green = decreased <0.6; ND = not detectable; blank = no change)

	E5 MucilAir T1	E5 MucilAir T5	E5 BEAS-B2 T1	E5 BEAS-B2 T5	E20 MucilAir T1	E20 MucilAir T5	E20 BEAS-B2 T1	E20 BEAS-B2 T5
Cytokines								
IL-1 alpha			ND	green			red	
IL-1 beta	ND	ND	ND	green	ND	ND	ND	ND
IL-1RA	red	red	ND	green	ND	red	ND	ND
IL-6	red	red	red	ND				
IL-7	ND		ND	ND	ND		ND	ND
TNF alpha	ND	red	ND	ND	ND	ND	ND	ND
Chemokines								
Eotaxin			ND	ND	red	red	ND	ND
GRO alpha								
IP-10		red	ND	green	red	red	ND	
IL-8	red			green				
MCP-1	red	red						
MIP-1 alpha	ND	red	ND	ND	ND	ND	ND	red
MIP-1 beta				green			red	
RANTES		red	ND	ND	red		ND	ND
SDF-1 alpha				green			red	
Growth factors								
BDNF	ND		ND	ND	ND	ND	ND	ND
EGF	ND	ND			ND	ND		
FGF2								
GM-CSF	red	red	ND	ND	ND	ND	ND	ND
LIF	red	red	ND	green	ND	ND	ND	
PIGF-1								
VEGF-A								

B

2B. E5 EOM / E20 EOM (red = increased >1.5; green = decreased <0.6; ND = not detectable; blank = no change)

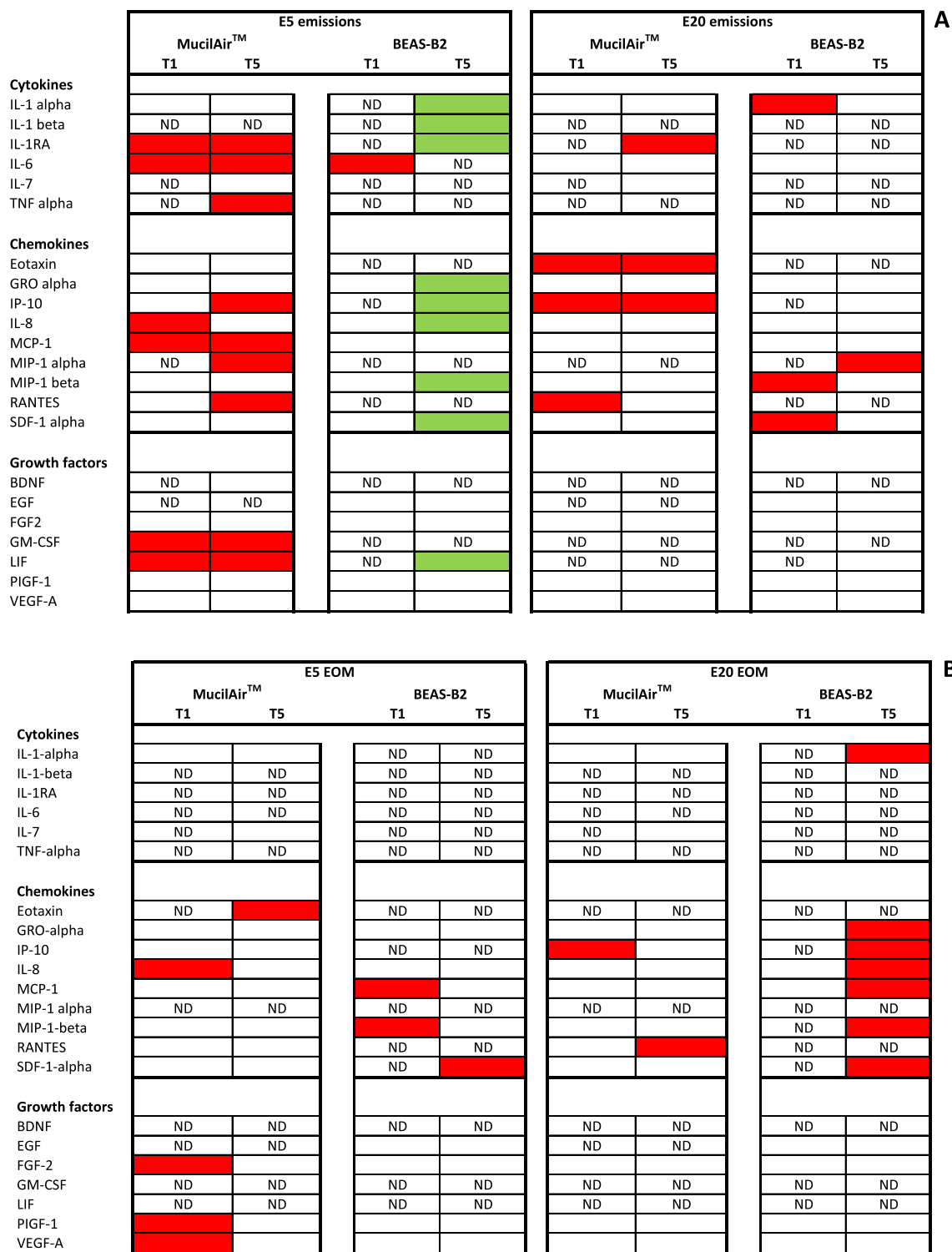|	E5 MucilAir T1	E5 MucilAir T5	E5 BEAS-B2 T1	E5 BEAS-B2 T5	E20 MucilAir T1	E20 MucilAir T5	E20 BEAS-B2 T1	E20 BEAS-B2 T5
Cytokines								
IL-1-alpha			ND	ND			ND	red
IL-1-beta	ND	ND	ND	ND	ND	ND	ND	ND
IL-1RA	ND	ND	ND	ND	ND	ND	ND	ND
IL-6	ND	ND	ND	ND	ND	ND	ND	ND
IL-7	ND		ND	ND	ND		ND	ND
TNF-alpha	ND	ND	ND	ND	ND	ND	ND	ND
Chemokines								
Eotaxin	ND	red	ND	ND	ND	ND	ND	ND
GRO-alpha								red
IP-10			ND	ND	red		ND	red
IL-8	red							red
MCP-1			red					red
MIP-1 alpha	ND	ND	ND	ND	ND	ND	ND	ND
MIP-1-beta			red				ND	red
RANTES			ND	ND		red	ND	ND
SDF-1-alpha			ND	red			ND	red
Growth factors								
BDNF	ND	ND	ND	ND	ND	ND	ND	ND
EGF	ND	ND			ND	ND		
FGF-2	red							
GM-CSF	ND	ND	ND	ND	ND	ND	ND	ND
LIF	ND	ND	ND	ND	ND	ND	ND	ND
PIGF-1	red							
VEGF-A	red							

Fig. 2. 2A. Production of immune response-relevant molecules by MucilAir™ and BEAS-2B cells exposed to complete emissions from E5 and E20 fuels at time points T1 and T5. Red and green colors denote increased (>1.5) and decreased (<0.6) levels of the respective marker, when compared with the control at the individual time point. ND, not detectable. **2B.** Production of immune response-relevant molecules by MucilAir™ and BEAS-2B cells exposed to EOMs from complete emissions from E5 and E20 fuels at time points T1 and T5. Both EOMs were prepared from a comparable amount of PM. Red and green colors denote increased (>1.5) and decreased (<0.6) levels of the respective marker, when compared with the control at the individual time point. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In contrast to complete emissions, EOM induced a more pronounced response in both cell models, although E5 EOM tended to decrease the levels of analyzed molecules, while E20 EOM exposure was rather associated with increased concentrations of lipid oxidation products. A relatively weak response was found in MucilAir™ exposed to E20 EOM. This EOM induced the levels of PGA$_2$, PGE$_2$ and 13, 14-DH-15-keto-PGE$_2$ in both cell models. The response of BEAS-2B cells was characterized by changes of concentrations of HETE molecules; in MucilAir™ this response was very weak, limited to exposure to E5 at the T5 time point. PGA$_2$ levels were affected by most of the exposure conditions in both cell models. A common response was further observed for 13, 14-dihydro-15-keto-PGE$_2$ and partly for 9-, 11-, 12- and 15-HETE for which, however, the response in MucilAir™ was limited to exposure to E5 EOM at T5 (Fig. 1B; Supplementary File 3).

Overall, PGA$_2$ and PGF$_{2\beta}$ were the most commonly affected molecules for both cell models and exposure conditions. The decrease of lipid oxidation product concentration was more frequently observed than the increase of the levels, this observation being driven by the effects of E5 EOM.

3.2. Cytokines, chemokines, and growth factors

The analyzed set of inflammatory markers consisted of 45 proteins, including 24 cytokines, 9 chemokines and 11 growth factors (Supplementary File 2). The levels of 23 molecules (51%) were not detectable for any of the tested conditions. Most of the non-detectable compounds (18) were cytokines, and 5 molecules belonged among growth factors. For the remaining 22 markers, we found a biological response, although the significant data was not obtained for all experimental conditions for any of the molecules (Fig. 2A and B; Supplementary File 4). The data indicate substantial differences, both between the cell models (MucilAir™ vs. BEAS-2B) and exposure conditions (complete emissions vs. EOM). In general, exposure to complete emissions resulted in changes of a greater number of markers than exposure to EOM (35 vs. 18 significant responses, for complete emissions and EOM, respectively). The most pronounced response was observed in cell cultures exposed to E5 emissions: while in MucilAir™ the exposure was associated with an increased production of all the significantly affected markers, in BEAS-2B cells the levels of such molecules decreased. In contrast, E20 emissions elicited an overall weaker response, particularly in BEAS-2B cells where a significant induction of levels of four markers was noted. Similar to E5 emissions, in MucilAir™ an increased production of analyzed molecules was detected after E20 exposure. Although for the majority of the parameters a unique response limited to a cell model/treatment condition was observed, IL-1RA levels were affected in MucilAIr™ by exposure to emissions from both fuels, while IL-6 production was modulated in both cell systems following exposure to E5 emissions. It was also noted that IP-10 production by MucilAir™ increased after exposure to E5 and E20 emissions, as well as by E20 EOM in both cell systems (Fig. 2A; Supplementary File 4).

The production of inflammatory markers after exposure to EOM increased. We observed elevated levels for 18 combinations of cell model/incubation condition. EOM from E5 emissions had a slightly greater impact on MucilAir™ when compared with BEAS-2B cells. In contrast, EOM from E20 emissions increased the levels of the analyzed markers in BEAS-2B cells, mostly at T5, while the response of MucilAir™ was minimal. A common response for both cell systems and/or tested EOM was observed for IP-10, MIP 1-beta, SDF 1-alpha and IL-8 (Fig. 2B; Supplementary File 4).

Overall, IP-10 was the molecule most commonly affected by the tested compounds in the investigated cell models. Increased production of the analyzed markers was detected for 44 combinations of the cell model/exposure condition, while decreased levels were found for 9 of such comparisons.

4. Discussion

Exposure to road vehicle emissions has a significant negative impact on human health and contributes to increased incidence of cardiovascular, pulmonary, and neurological diseases, as well as cancer. Chronic inflammation has been shown to play an important role in the progression of these disorders (Manzetti and Andersen, 2016). In our study we investigated a link between the exposure of bronchial epithelial cells to gasoline emissions and the production of pro-inflammatory molecules – lipid oxidation products and immunomodulatory proteins. While the biological response induced by complete emission exposure is associated with particles, chemical components bound to them, and gaseous pollutants, EOMs exclusively mediate their effects via organic constituents, including PAHs. PAHs are known to induce an inflammatory response (Vogel et al., 2020) resulting in the production of AA metabolites. The final response of the exposed cells thus depends on a combination of biological activities of the gasoline emission components and their bioavailability. The EOM exposure provides more direct contact of the cell cultures with the tested compounds, than exposure to complete emissions where the diffusion of the exhaust components from the gas phase via culture media to the cells is required; thus reducing the effective dose of the emissions. However, this situation is more reflective of the processes in human lungs, making the exposure to complete emissions a more realistic setting than EOM treatment.

4.1. Lipid oxidation products

Despite their important biological role, lipid oxidation products have not been thoroughly investigated in the context of exposure to gasoline engine emissions, and there is a lack of studies comparable with our work. In this study, we assessed the formation of 22 lipid oxidation products by two cell models exposed to complete gasoline emissions and EOMs. While we detected changes of the levels of 16 of the selected compounds, none of the products were commonly affected by all of the exposure conditions. Considering the differences in chemical composition between complete emissions and EOMs, such an observation could be expected.

The exposure scenarios used in our study resulted in both increased and decreased levels of lipid oxidation products, although the overall response was relatively weak. Assuming the pro-inflammatory effects of the majority of these compounds, their elevated levels reflect the toxicity of gasoline emissions and EOMs. However, some PG and their derivatives may exert anti-inflammatory reactions. Therefore, the overall response could be very complex and the potential biological impacts should be cautiously interpreted. In general, an increased production of the analyzed compounds was rather detected after exposure to complete E5 emissions and treatment with EOM from E20, while lower levels of lipid peroxidation products were linked with E5 EOM exposure.

The production of PGF$_{2\beta}$ was predominantly associated with complete emission exposure, notably after E5 treatment in MucilAir™, where elevated levels were detected at both time points. Limited information can be found in literature on the biological functions of this PG. A report from the year 1973 indicates the bronchodilating effects it has in guinea pigs and cats, suggesting the potentially negative biological effects in human lungs associated with this molecule (Rosenthale et al., 1973). PGE$_2$ and PGF$_{2\alpha}$ were also produced after complete E5 emission exposure, although the response was limited to MucilAir™ at the T1 time point. While PGF$_{2\alpha}$ plays a role in acute and chronic inflammatory diseases (Basu, 2010), anti-inflammatory effects of PGE$_2$ in the lungs have been reported (Vancheri, 2004). In BEAS-2B cells, the production of 5-HETE, 8-HETE, 9-HETE and 11-HETE was detected. The pro-inflammatory effects of these molecules have been described (Kiss et al., 2000; Liu et al., 2014); increased levels were also observed in lung tumors (Liu et al., 2014). In our study, EOM exposure had mostly inhibitory effects on HETE production, particularly after E5 EOM

treatment.

The production of PGA$_2$ was almost exclusively increased after E20 EOM exposure, specifically by BEAS-2B cells. Overall, this molecule, along with PGF$_{2\beta}$, was the most commonly affected in our study. PGA$_2$ that belongs among the cyclopentenone prostaglandins has anti-neoplastic effects, similar to PGE$_2$, also detected after E20 EOM treatment (Straus and Glass, 2001). The decreased production of this molecule, observed e.g. after exposure to E5 EOM, may suggest a potentially decreased antitumor protection in the exposed cells. Similarly to PGA$_2$, the production of 13, 14-dihydro-15-keto-PGE$_2$, a stable metabolite of PGE$_2$, was increased after E20 EOM treatment rather than after other exposure conditions. This compound has been shown to induce the production of oncostatin M, a cytokine with anti-inflammatory activity (Ganesh et al., 2012). Thus, its overproduction may be associated with the induction of an anti-inflammatory response after E20 EOM exposure. The activity of another PG, PGD$_2$, is linked with chronic inflammation, as it is predominantly released by cells that play a role in the patho-physiology of asthma (Domingo et al., 2018). The production of this molecule was induced by EOM E20 treatment in BEAS-2B cells, but a decrease was observed for other exposure conditions.

Overall, E5 emission exposure tended to mediate a pro-inflammatory response in MucilAir™; in BEAS-2B cells a mixed response of pro- and anti-inflammatory molecules can be observed. For the EOM from E5, mostly decreased levels of pro-inflammatory molecules were detected, suggesting minor effects of organic components in E5 EOM on the induction of a lipid oxidation cascade. The discrepancy in the data obtained for MucilAir™ and BEAS-2B cells, may be attributed to the fundamental physiological and histological differences between the cell models. While BEAS-2B cells are an adherent cell line derived from normal bronchial epithelium immortalized by a virus, MucilAir™ represents fully differentiated bronchial epithelial cells reconstituted from healthy tissue that consists of human basal, goblet, and ciliated cells and displays in vivo characteristics, such as stratification, tight junctions, mucus production or cilia beating (Huang et al., 2013). Interestingly, exposure to E20 EOM increased the production of several PG with anti-inflammatory properties, indicating a low inflammation-related response of this extract. This result is unexpected, as exposure to PAHs has been shown to induce chronic inflammation (Vogel et al., 2020) and the PAH content was overall higher in E20 than in E5 EOM (e.g. a 60% difference for carcinogenic PAHs). We may speculate that further, not as yet identified components of E20 EOM, exert effects that may cause a limited pro-inflammatory response.

Finally, we should mention that levels of 8-Iso-PGF$_{2\alpha}$ were not modulated by any exposure conditions. This molecule, also known as 15-F$_{2t}$-isoprostane, is regarded as a widely accepted marker of oxidative stress (Milne et al., 2015). Thus, oxidative damage after gasoline emission exposure seems to play a minor role in our study. In agreement with these data, we have previously reported the limited pro-oxidant activities of PAHs and EOMs in model lung cell lines (Libalova et al., 2018; Rossner et al, 2019b, 2020). In another study, the effects of EOM from PM collected from urban sources (including gasoline cars) on a lipid peroxidation marker (malondialdehyde) were investigated. The authors did not observe any induction of malondialdehyde levels after gasoline PM exposure, also indicating the low pro-oxidant properties of these compounds (Velali et al., 2018).

4.2. Immunomodulatory proteins

Most studies focused on the immune response after engine emission exposure, have investigated the effect of diesel fuels. Research on the *in vitro* toxicity of gasoline emissions in model cell lines was reported by a limited number of publications that did not observe any significant induction of pro-inflammatory response. Thus, the exhaust from gasoline containing 0%, 10% and 85% ethanol was used to treat a model consisting of human bronchial cells, dendritic cells, and macrophages. The cell cultures were exposed for 6 h using a steady state cycle, and toxicity

markers were assessed after a 6 h post-incubation period. The authors did not detect a significant induction of genes encoding pro-inflammatory molecules (TNFα and IL-8) or other toxicity markers, concluding that the adverse effects of gasoline emissions are minimal (Bisig et al., 2016). In another *in vitro* study, aged gasoline exhaust particles had hardly any effect on the production of IL-6, IL-8 and MCP-1 in HBE and BEAS-2B cells after a short, acute exposure, suggesting an impairment of defense mechanisms and higher vulnerability to subsequent adverse conditions (Künzi et al., 2015).

Contrary to these reports, we performed a comprehensive analysis of immune response-related molecules that included a panel of 45 cytokines, chemokines, and growth factors and exposed the cells for an extended period of time. The levels of 22 of these proteins were found to be affected by complete gasoline emissions and/or EOM treatment. Similarly to lipid oxidation products, the response was usually cell model/exposure condition-specific. Overall, complete E5 emissions elicited the most pronounced effects, particularly in MucilAir™, where the increased production of 15 markers was detected. They included cytokines, such as interleukin (IL) 1 receptor agonist (IL-1RA; a regulator of pro-inflammatory cytokines IL-1 alpha and IL-1 beta (Fields et al., 2019)), IL-6 (a key molecule in acute and chronic inflammatory response (Gabay, 2006)), tumor necrosis factor alpha (TNF alpha; a regulator of inflammatory cytokine production (Parameswaran and Patial, 2010)); chemokines, e.g. interferon gamma-induced protein 10 (IP-10; a molecule inducing chemotaxis, apoptosis or cell growth (Liu et al., 2011), IL-8 specifically targeting neutrophils (Bickel, 1993), monocyte chemoattractant protein-1 (MCP-1; a chemokine that regulates the migration and infiltration of monocytes/macrophages (Deshmane et al., 2009)), macrophage inflammatory protein 1 alpha (MIP-1 alpha; a molecule that recruits inflammatory cells (Bhavsar et al., 2015)), regulated upon activation, normal T cell expressed and presumably secreted (RANTES; a chemokine with a variety of functions, including the induction of inflammation (Marques et al., 2013)) and growth factors (granulocyte-macrophage colony stimulating factor (GM-CSF; a pro-inflammatory molecule that acts as a growth and differentiation factor for granulocytes and macrophages (Bhattacharya et al., 2015), and a leukemia inhibitory factor (LIF), an important molecule in chronic airway inflammation (Knight, 2001)). In BEAS-2B cells the emissions mainly caused a decreased production of inflammatory molecules, especially at time point T5. Considering our data on the biological response of this cell line after E5 emission exposure (Rossner et al., 2019a) this result most likely reflects the toxicity, rather than "anti-inflammatory" properties of E5 emissions in this cell line. E20 emissions elicited a generally weaker inflammation-related response, mostly limited to MucilAir™. In contrast to E5, the exhaust from this fuel affected the production of eotaxin, that is known to play a role in airway inflammation (Adar et al., 2014).

The effects of exposure to EOM from both tested fuels on markers of inflammation were generally minimal with most of the positive results limited to chemokines, especially after treatment of BEAS-2B cells with E20 EOM at time point T5. Apart from the afore-mentioned IP-10 and MCP-1 molecules, these conditions induced the production of growth-regulated oncogene (GRO alpha), macrophage inflammatory protein 1 beta (MIP-1 beta) and stromal cell-derived factor 1 alpha (SDF-1 alpha), chemokines that are implicated in inflammatory lung diseases and lung injury (Capelli et al., 1999; Traves, 2002; Xu et al., 2007).

5. Conclusions

In summary, our study showed differences in the pro-inflammatory response in bronchial epithelial cell models, after exposure to two types of gasoline complete emissions and EOMs. The results suggest that the inflammation-related toxicity of ordinary gasoline is substantially greater than that of the alternative E20 fuel. The response is linked rather with particles and/or gaseous components of the exhaust, than with the organic compounds bound to particles. The effects of the

alternative fuel were generally weaker, mostly associated with the organic fraction bound to the particles. In conclusion, our data indicate that ethanol-gasoline blends may be used as a suitable alternative to ordinary gasoline, as they mediate lower immunotoxicity and thus represent a lower risk of inflammatory respiratory diseases.

Credit author statement

Pavel Rossner Jr, Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Supervision; Roles/Writing – original draft; Writing – review & editing. Tereza Cervena, Investigation; Writing – review & editing. Michal Vojtisek-Lom, Funding acquisition; Writing – review & editing. Jiri Neca, Investigation; Writing – review & editing. Miroslav Ciganek, Investigation; Writing – review & editing. Kristyna Vrbova, Investigation; Writing – review & editing. Antonin Ambroz, Investigation; Writing – review & editing. Zuzana Novakova, Investigation; Writing – review & editing. Fatima Elzeinova, Investigation; Writing – review & editing. Michal Sima, Formal analysis; Visualization; Writing – review & editing. Zuzana Simova, Visualization; Writing – review & editing. Vladimir Holan, Writing – review & editing. Vit Beranek, Investigation; Writing – review & editing. Martin Pechout, Investigation; Writing – review & editing. David Macoun, Investigation; Writing – review & editing. Andrea Rossnerova, Writing – review & editing. Jan Topinka, Writing – review & editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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