TOXIC RESPONSES IN HUMAN LUNG EPITHELIAL CELLS (BEAS-2B) EXPOSED TO PARTICULATE MATTER EXHAUST EMISSIONS FROM GASOLINE AND BIOGASOLINE

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Abstract

Motor vehicle emissions substantially contribute to air pollution worldwide and cause serious health problems. While the deleterious effects of diesel exhaust particulate matter (PM) have been widely studied, much less attention is paid to toxicity of PM emitted by gasoline engines although they also produce considerable amount of PM. The primary objective of this research was to assess toxic potencies of exhaust PM released by conventional gasoline engine fueled with neat gasoline (E0) or gasoline-ethanol blend (15% ethanol, v/v, E15). Despite a similar particle mass (µg PM/kg fuel) produced by both fuels, PM emitted by E15 contained higher amount of harmful polycyclic aromatic hydrocarbons (PAH) as suggested by chemical analysis. To examine the toxicity of organic PM constituents, human lung BEAS-2B cells were exposed for 4h and 24h to a subtoxic dose of E0 and E15 PM organic extracts. We used genome scale transcriptomic analysis to characterize the toxic response and to identify modulated biological process and pathways. Whereas 4h exposure to both PM extracts resulted in modulation of similar genes and pathways related to lipid and steroid metabolism, activation of PPARα, oxidative stress and immune response, 24h exposure was more specific for each extract; although both induced expression of PAH-metabolic enzymes, modulated metabolism of lipids or activated PPARα, E15 additionally deregulated variety of other pathways. Overall, the PM mass produced by both fuels was similar, however, higher PAH content in E15 PM organic extract may have contributed to more extensive toxic response particularly after 24h exposure in BEAS-2B cells.

Keywords: Particulate matter emissions, gasoline, biofuels, toxicity, gene expression profiling

1. INTRODUCTION

Motor vehicle emissions substantially contribute to air pollution worldwide. They represent an important source of particulate matter (PM). Numerous scientific studies have linked PM exposure to various health effects including pulmonary and cardiovascular disorders or cancer [1]. Combustion-related PM consists of relatively

high concentrations of toxic substances bound on the particle surface. Among them, polycyclic aromatic hydrocarbons (PAHs) play an important role in PM toxicity [2]. Of special concern are ultrafine particles (<100 nm) because they can deposit deep into the lungs, enter the bloodstream and reach other organs [3]. Compared to coarse (>2,5 μm) or fine (100 nm-2,5 μm) PM, ultrafine particles exhibit a large surface area that allows them to carry a relatively large load of toxic compounds. For many years, diesel engines dominated among the mobile sources responsible for negative environmental issues and adverse health effects associated with PM exposure. However, modern gasoline engines recently emerged as important contributors to traffic related particulate pollution [4]. Increasing demands for the replacement of conventional fossil fuels with alternatives produced from renewable sources have raised an interest in numerous biofuels and their blends. Ethanol is the most commonly used bio additive for gasoline. Despite this fact, studies on the comparative toxicity of particulate emissions or their organic extracts from fossil gasoline and gasoline-ethanol blends are still scarce. The present study aimed to compare the toxic effects of organic complex mixtures extracted from particulate emissions produced by a passenger car fueled with neat gasoline (E0) or ethanol-gasoline blend (E15). To achieve this, we employed genome-scale mRNA expression profiling as a sensitive method to monitor the complex molecular response and thus reveal deregulated genes, processes and pathways.

2. METHODS

2.1. PM collection, extraction of organic compounds and chemical analysis

A 2011 Skoda Fabia 1.4 16V, 5MT, MPI was driven on four-wheel chassis dynamometer MAHA AIP-ECDM 48L-4mot along basic driving cycle the Common Artemis Driving Cycle (motorway variant 130 km.h-1). The exhaust has been routed into a fullflow dilution tunnel with a constant volume sampler (CVS) operating at 10.8 m3.min-1. Particles were sampled on teflon-coated glass fiber filters (Pall TX40HI20-WW), at 67.8 m3.h-1 sampling rate. Organic compounds were extracted with dichloromethane and both E0 and E15 extracts were subjected to chemical analysis and PAH contaminants were quantified. The procedure is described elsewhere [5].

2.2. Cell cultures, exposure conditions, RNA isolation and microarray analysis

BEAS-2B cells were exposed to 50 μg/mL of each extract for 4 and 24h. Cell viability was assessed using WST-1 and LDH assays. RNA from cell lysates was extracted and the integrity of RNA was analyzed using Agilent 2100 Bioanalyzer. RNA was then transcribed into biotinylated complementary DNA and hybridized onto Human-HT12 v4 Expression BeadChips (Illumina). The detailed protocol is presented in [5].

3. RESULTS

3.1. Characteristics of collected gasoline PM and chemical analysis of organic extracts

Total particulate mass emissions (expressed in μg per kg of fuel) produced by the tested vehicle was comparable for both E0 and E15 fuels. However, the number of particles > 20 nm was app. 40 times higher for E15 than for E0 and also chemical analysis revealed that the sum of all analyzed PAHs and their derivatives such as oxy-, nitro- or dinitro-PAHs in E15 extract was several fold higher than in E0 extract (**Table 1**).

Table 1 PM characteristics and PAH content in fuel extracts

	E0	**E15**
PM (μg/kg of fuel)	112,2	110,3
Particle number (per driving cycle)	$6{,}23 \times 10^5$	$2{,}56 \times 10^7$
Sum of PAHs (ng/mg PM)	579,0	1944,2
Sum of cPAHs (ng/mg PM)	203,5	887,6
Sum of oxygenated PAHs (ng/mg PM)	368,4	477,9
Sum of nitrated PAHs (pg/mg PM)	1117,8	684,0
Sum of dinitrated PAHs (pg/mg PM)	21,1	14,7

3.2. Gene expression profiling

3.2.1. Differential expression of individual genes

Four-hour incubation of BEAS-2B cells with both extracts resulted in deregulation of 13 common genes while 5 deregulated genes were specific for E0 and 22 genes for E15 treatment (**Figure 1A**). After 24h exposure, we found 42 common significantly deregulated genes, 29 genes specific for E15 and 77 genes for E0 treatment (**Figure 1B**).

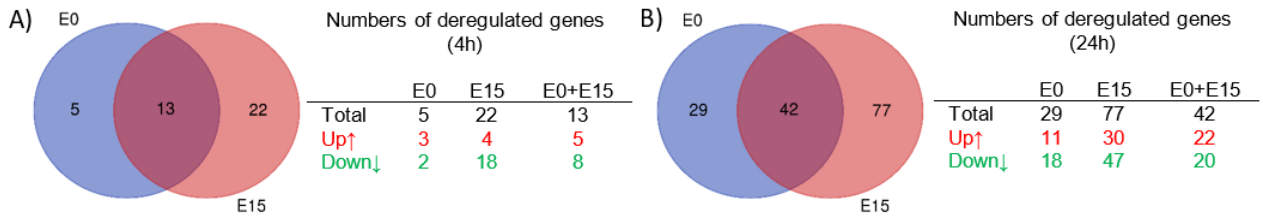

Figure 1 Venn diagrams illustrating the number of significantly deregulated genes for E0 and E15 and their overlap after A) 4h and b) 24h exposure (p-value < 0.05, fold change > 1.5 and < 0.67)

3.2.2. Pathway analysis

In order to reveal modulated biological processes and signaling pathways, we analyzed differentially expressed genes using ToppFun, a tool providing transcriptome, ontology, phenotype, proteome and pharmacome annotations based gene list functional enrichment analysis (https://toppgene.cchmc.org/). After 4h exposure, both extracts deregulated a variety of pathways related to cellular lipid metabolism and homeostasis, steroid hormone biosynthesis or Sterol Regulatory Element Binding Protein (SREBP) signaling. We further identified modulated pathways associated with Peroxisome proliferator-activated receptor alpha (PPARα) signaling or pathways indicating oxidative stress response and alterations in immune functions (**Table 2**). Each individual extract treatment also affected a range of specific pathways (**Table 3** and **Table 4**).

Table 2 Top 5 common deregulated pathways with contributing genes after 4h exposure to both extracts

Pathway	Source (Biosystem)	E0	E15
Metabolism of lipids and lipoproteins	REACTOME	↑*TXNRD1, AKR1C2* ↓*LDLR, CTGF, COQ5, HMGCS1, INSIG1*	↑*TXNRD1, AKR1C2* ↓*LPIN1, CTGF, HMGCR, HMGCS1, IDH1, SC5DL, LDLR, INSIG1, SQLE, NSDHL*
Regulation of cholesterol biosynthesis by SREBP (SREBF)	REACTOME	↓*HMGCS1, INSIG1*	↓*HMGCR, HMGCS1, SC5DL, INSIG1, SQLE*
NFkB activation by Nontypeable Hemophilus influenzae	MSigDB C2 BIOCARTA (v6.0)	↓*IL8, NFKBIA*	↓*TGFBR2, IL8, NFKBIA*
Validated transcriptional targets of AP1 family members Fra1 and Fra2	Pathway Interaction Database	↑*TXNRD1* ↓*CTGF, HMGCS1*	↑*HMOX1, FOSL1* ↓*IL8*
PPARA activates gene expression	REACTOME	↑*HMOX1* ↓*IL8*	↑*TXNRD1* ↓*CTGF, HMGCR, HMGCS1*

Table 3 Top five specific deregulated pathways with contributing genes after 4h exposure to E15 extract

Pathway	Source (Biosystem)	E15
cholesterol biosynthetic	Pathway Ontology	↓*HMGCR, HMGCS1, SC5DL, SQLE, NSDHL*
mevalonate pathway	BIOCYC	↓*HMGCR, HMGCS1*
IL-17 signaling pathway	KEGG	↑*FOSL1* ↓*IL8, NFKBIA*
NADPH regeneration	REACTOME	↓*IDH1*
Fluid shear stress and atherosclerosis	KEGG	↑*HMOX1, CTSL1* ↓*KLF2*

Table 4 Top five specific deregulated pathways with contributing genes after 4h exposure to E0 extract

Pathway	Source (Biosystem)	E0
Ferroptosis	KEGG	↑*GCLM, HMOX1*
Fas Signaling Pathway	MSigDB C2 BIOCARTA (v6.0)	↓*IL8, NFKBIA*
LPA receptor mediated events	Pathway Interaction Database	↓*IL8, NFKBIA*
Glutathione biosynthesis, glutamate => glutathione	KEGG	↑*GCLM*
Atherosclerosis	Pathway Ontology	↓*LDLR*

Twenty-four hour exposure resulted in more distinct expression profiles. Whereas several pathways deregulated by both extracts were similar to those modulated after 4h exposure (lipid metabolism, SREBP signaling, PPARα signaling), others (allopregnanolone biosynthesis, interleukin-1 processing) were specific for 24h exposure only (**Table 5**). E0 specifically modulated pathways related to metabolism of steroids and xenobiotics (**Table 6**) while E15 rather affected a large variety of pathways linked to lipid and sterol metabolism, extracellular matrix assembly and organization, cell junction and communication or MAPK signaling associated with cellular stress (**Table 7**).

Table 5 Top 5 common deregulated pathways with contributing genes after 24h exposure to both extracts

Pathway	Source (Biosystem)	E0	E15
Steroid hormone biosynthesis	KEGG	↑*CYP1A1, CYP1B1, AKR1C4, AKR1C2*	↑*CYP1B1, AKR1C4, AKR1C2* ↓*HSD17B8*
Metabolism of lipids and lipoproteins	REACTOME	↑*TXNRD1, MED24, CYP1A1, CYP1B1, AKR1C4, AKR1C2, ANGPTL4* ↓*PPARGC1A, TM7SF2, FADS2, LSS, COL4A3BP*	↑*AKR1C4, AKR1C2, MED24, ANGPTL4, CYP1B1* ↓*TECR, HMGCR, HMGCS1, NSDHL, LPIN1, PPARGC1A, FDFT1, SLC25A1, EBP, TM7SF2, LSS, MVD, DBI, HSD17B8, PCSK9*
PPARA activates gene expression	REACTOME	↑*TXNRD1, MED24, CYP1A1, ANGPTL4* ↓*PPARGC1A*	↑*MED24, ANGPTL4* ↓*HMGCR, HMGCS1, PPARGC1A, FDFT1*
allopregnanolone biosynthesis	BIOCYC	↑*AKR1C4, AKR1C2*	↑*AKR1C4, AKR1C2*
Interleukin-1 processing	REACTOME	↑*IL1A, IL1B*	↑*IL1A, IL1B*

Table 6 Top specific deregulated pathways with contributing genes after 24h exposure to E15 extract

Pathway	Source (Biosystem)	E15
Fatty acid, triacylglycerol, and ketone body metabolism	REACTOME	↑*MED24, ANGPTL4* ↓*TECR, HMGCR, HMGCS1, LPIN1, PPARGC1A, FDFT1, SLC25A1, DBI, HSD17B8*
Assembly of collagen fibrils and other multimeric structures	REACTOME	↑*LAMA3, LAMB3, LAMC2, CTSL1* ↓*COL8A1*
Laminin interactions	REACTOME	↑*LAMA3, LAMB3, LAMC2*
Degradation of the extracellular matrix	REACTOME	↑*LAMA3, LAMB3, LAMC2, CTSL1* ↓*CDH1*
MAPK signaling pathway	KEGG	↑*MYC, RPS6KA2, DUSP6, IL1A, IL1B* ↓*STMN1, FGFR3*

Table 7 Top specific deregulated pathways with contributing genes after 24h exposure to E0 extract

Pathway	Source	E0
bioactivation via cytochrome P450	Pathway Ontology	↑*CYP1A1, CYP1B1*
Synthesis of epoxy (EET) and dihydroxyeicosatrienoic acids (DHET)	BioSystems: REACTOME	↑*CYP1A1, CYP1B1*
Synthesis of (16-20)-hydroxyeicosatetraenoic acids (HETE)	BioSystems: REACTOME	↑*CYP1A1, CYP1B1*
gamma-hexachlorocyclohexane degradation	Pathway Ontology	↑*CYP1A1, CYP1B1*

4. DISCUSSION

Our results demonstrated that the mass of particulate emissions was not substantially affected by the supplementation of gasoline fuel with 15% ethanol. This is in line with our previous study [5] where we observed similar PM mass emitted by E0 and E15 although different engine technology (GDI) was used. On the other hand, in the recent study [6], authors reported that ethanol rather reduced the mass of PM. Such inconsistency might be caused by many factors including differences among engine design, calibration, settings and operating conditions. Importantly, despite similar particle mass, E15 produced much higher particle number comparing to E0 indicating generation of harmful ultrafine particles (<100 nm). This finding correlates with the higher concentration of PAHs in E15 organic extract as revealed by chemical analysis. Gene expression profiling revealed numerous processes similarly deregulated processes after 4h exposure to both E0 and E15 extract. Suppression of lipid metabolism and cholesterol synthesis was evident due to the downregulation of key contributing genes HMGCR, HMGCS1 and others. It has been demonstrated that PAHs repress genes involved in cholesterol biosynthesis and disrupt lipid homeostasis through an activated aryl hydrocarbon receptor (AhR) [7]. Both extracts further triggered oxidative stress response, possibly due high amount of ROS released during metabolism of PAHs or by other redox-active organic compounds. HMOX1 and TXNRD1 are key redox-sensitive enzymes that neutralize ROS and help to maintain redox homeostasis. Both enzymes are regulated by NRF2 transcription factor that controls a variety of processes responsible for cellular defense against xenobiotic and oxidative stress [8]. Oxidative stress and antioxidant response may also negatively regulate expression and activity of PPARα [9] as also indicated by our results. Specific effects of E15 extract treatment involve downregulation of IDH1, one of the key enzymes in NADPH regeneration with the citrate cycle. It has been shown that PAHs also involve alterations of mitochondrial functions and decrease gene expression of mitochondrial enzymes including IDH1 in multiple organs [10]. Twenty four hour exposure was characterized by metabolism of PAHs and activation of AhR. Upregulation of CYP1B1, AKR1C2 and AKR1C4 was common for both extracts while expression of CYP1A1 was elevated after E0 extract treatment only. CYP1 enzymes participate in the metabolic activation of PAHs and formation of reactive intermediates which form stable DNA adducts. Other enzymes, such as AKRs, are also implicated in PAH activation and generate redox-active PAH *o*-quinones accompanied with formation of ROS [11]. Similarly as for 4h exposure, suppressed metabolism of lipids and cholesterol as well as PPARα signaling was observed. Increased expression of IL1A and IL1B indicated immune response associated with genotoxic and oxidative stress [12]. In contrast to E0, E15 extract modulated a variety of pathways related to extracellular matrix. The role of PAHs in extracellular matrix remodeling has been proposed [13]. E15 further activated MAPK signaling which is known to regulate various cell functions such as proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis in response to diverse stimuli including oxidative and genotoxic stress. It has been suggested that PAHs in nanomolar levels are able to induce MAPKs [14].

5. CONCLUSIONS

Although E0 produced similar mass of PM as E15, the number of particles was much higher for E15 than for E0 indicating generation of particularly toxic ultrafine particles. Accordingly, chemical analysis revealed a higher content of PAHs in E15 extract. The toxic effects of E0 and E15 extracts in BEAS-2B cells were mostly associated with the action of PAHs and activated AhR. Four-hour incubation with both extracts resulted in many identically deregulated processes, such as suppression of lipid metabolism and PPARα signaling or oxidative stress response. On the other hand, response to 24h exposure was more diverse, besides common processes, such as activation of AhR and metabolism of PAHs, suppressed lipid metabolism, activation of PPARα- or IL1-dependent immune response, E15 extract specifically induced a large variety of pathways related to extracellular matrix assembly or MAPK signaling. In contrast, only few specific pathways were found after 24h treatment with E0 extract. Taken together, E15 extract elicited a more extensive toxic response particularly after 24h treatment than E0 extract, possibly due to the higher content of PAHs. These findings may help to explore the impact of PM emissions generated by alternative gasoline fuels on human health.

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