

Effect of diesel exhaust generated by a city bus engine on stress responses and innate immunity in primary bronchial epithelial cell cultures



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ABSTRACT

Harmful effects of diesel emissions can be investigated *via* exposures of human epithelial cells, but most of previous studies have largely focused on the use of diesel particles or emission sources that are poorly representative of engines used in current traffic.

We studied the cellular response of primary bronchial epithelial cells (PBECs) at the air-liquid interface (ALI) to the exposure to whole diesel exhaust (DE) generated by a Euro V bus engine, followed by treatment with UV-inactivated non-typeable *Haemophilus influenzae* (NTHi) bacteria to mimic microbial exposure. The effect of prolonged exposures was investigated, as well as the difference in the responses of cells from COPD and control donors and the effect of emissions generated during a cold start. *HMOX1* and *NQO1* expression was transiently induced after DE exposure. DE inhibited the NTHi-induced expression of human beta-defensin-2 (*DEFB4A*) and of the chaperone *HSPA5/BiP*. In contrast, expression of the stress-induced *PPP1R15A/GADD34* and the chemokine *CXCL8* was increased in cells exposed to DE and NTHi. *HMOX1* induction was significant in both COPD and controls, while inhibition of *DEFB4A* expression by DE was significant only in COPD cells. No significant differences were observed when comparing cellular responses to cold engine start and prewarmed engine emissions.

1. Introduction

Diesel exhaust (DE) exposures constitute a health risk for large populations living in urbanized areas (Laumbach and Kipen, 2012; Schmidt, 2016). Especially those suffering from chronic inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma may experience an increase in exacerbations during periods of high diesel exhaust exposure (Andersen et al., 2011; Weinmayr et al., 2010). The mechanisms by which traffic-related air pollution may trigger exacerbations are largely unresolved. Oxidative stress is often considered a hallmark for DE exposure and is increased in chronic inflammatory lung disease (Barnes, 2016; Dozor, 2010). Indeed, several studies analyzed heme oxygenase 1 (*HMOX1*) and NAD(P)H quinone dehydrogenase 1 (*NQO1*) mRNA expression to characterize the DE-induced cellular oxidative stress (Hawley et al., 2014; Yamamoto et al.,

2013; Zarcone et al., 2016). However, a direct link between such DE-induced oxidative stress markers with disease exacerbations has not been established. In addition to air pollution, viral and bacterial infections are associated with COPD exacerbations (Vestbo et al., 2013). Non-typeable *Haemophilus influenzae* (NTHi) is commonly detected during acute COPD exacerbations, but is also present in patients with stable disease (Sethi and Murphy, 2008). Therefore, possibly DE exerts its effect on exacerbations through impairment of host defense against respiratory infections. The lung epithelium releases antimicrobial peptides and proteins such as human beta defensin (hBD)-2 and S100 calcium binding protein (S100A7) in response to bacterial infections (Hiemstra et al., 2015). Previously, impairment of such defenses by cigarette smoke was demonstrated and implicated in the increased susceptibility of smokers to respiratory infection (Herr et al., 2009; Pace et al., 2012). We have recently obtained evidence that DE may exert

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similar effects of epithelial cell cultures, as shown by its ability to decrease induced hBD-2 expression (Zarcone et al., 2017). Another mechanism that may deregulate epithelial defense in COPD is provided by the observation of chronic activation of the integrated stress response (ISR) in COPD and other inflammatory lung diseases that may result from oxidants such as cigarette smoke as well as micro-organisms (Steiling et al., 2013; van 't Wout et al., 2014). Therefore, detrimental effects of chronic activation of the ISR may also contribute to the effects of DE.

Air-liquid interface (ALI) cultures are a useful tool to investigate effects of exposure to different kind of toxic nanoparticles (Frieke Kuper et al., 2015; Kooter et al., 2013; Kooter et al., 2017). DE exposure of primary bronchial epithelial cells (PBEC), differentiated and exposed at the air-liquid interface (ALI) can better mimic the physiological composition of the lung epithelium *in vitro*. Effects of DE exposures on the antimicrobial response and the ISR of ALI-PBECs have been previously showed (Zarcone et al., 2016; Zarcone et al., 2017). Although these studies provided evidence for modulation of these mechanisms by DE, like most other studies they relied on the use of diesel exhaust emissions that can be compared to relatively high levels of ambient pollution. However, this may not fully represent the modern diesel engines that increasingly appear in traffic. Therefore, in the present study DE exposures were performed using emissions of city bus with Euro V diesel engine with selective catalytic reduction (SCR) catalyst using urea as reductant as after-treatment device, following the city-like Braunschweig testing cycle to mimic the pattern of acceleration typical for city traffic. We studied the cellular response of cells from (ex)smokers with a normal lung function and those with COPD to DE, and explored the modulation of bacteria-induced gene expression by DE to study modulation of innate immunity. Finally, we investigated the effect of DE generated by a cold engine (cold start) and compared this to that of a pre-heated engine.

2. Methods

2.1. Exposure to whole diesel exhaust generated by a Euro V bus engine

A typical Euro V bus engine (Cummins ISL 8.9 E5 280B) with maximal power of 209 kW was used at the TNO Automotive Powertrain Test Center (Helmond, the Netherlands) to produce diesel exhaust for all exposure sessions that were performed in a temporary biological research laboratory that was installed onsite. The 6 cylinders engine, with a cylinder displacement of 8.9 L, was connected to a selective catalytic reduction (SCR) device with urea dosing (after treatment device); no exhaust gas recirculation (EGR) system was present on the engine. During each exposure, the engine ran using commercially available (trade) fuel following an adapted Braunschweig city cycle (30 min per cycle), which is a typical bus city cycle (stop and go) that is commonly used for calibration purposes (Barlow et al., 2009). In addition, before the start of the experiment, the European transient cycle (ETC) test was performed to verify that the engine complies with the Euro V legislation. Experimental exposures were performed using a “warm” start set-up, following a first cycle of 30 min (“cold” start) during which the cells were not exposed. This design was followed to stabilize the emission and bypass a first burst of particulate matter (PM). In selected experiments, the effect of the cold start, in which particle concentrations were ~30% higher compared to normal operation (Fig. 1), was specifically addressed.

The diesel exhaust produced was first diluted in the constant volume sampler (CVS) tunnel with a constant volume flow of 3×10^4 L/min. The first dilution of DE (high, about 17 times diluted) was then collected in a central tank, to be directly addressed to the first Vitrocell® (Waldkirch, Germany) exposure module, or further diluted 2.5 or 6 times to generate the mid and low DE exposure conditions. Dilution of the DE was achieved by extracting DE from the central tank and adding mass flow controlled amounts of filtered humidified air. This exposure system

allowed exposure of three inserts to the same condition at 37 °C with a flux velocity of about 5 mL/min. In the central tank, the exhaust temperature (T) and relative humidity (RH) were measured with a RH/T device (TESTO 635-1, TESTO GmbH & Co, Lenzkirch, Schwarzwald, Germany) to be 25.5 ± 1.11 °C and $58.6 \pm 7.8\%$. The oxygen (O₂) level in the central tank was 19.9 ± 0.03 vol% (mean value from two prolonged exposure sessions) as measured using an M&C PMA-10 oxygen analyzer (M&C Products Analysetechnik GmbH, Ratingen-Lintorf, Germany). On average, the carbon dioxide (CO₂) level in the central tank was $0.152 \pm 0.02\%$ as measured using a Vaisala GM70 probe with MI70 read-out unit (Vaisala, Helsinki, Finland).

Chemical characterization of the emissions has been performed as described previously (Jedynska et al., 2015). In brief in the first stage sampling points were used for: CO, CO₂, NO_x and total hydrocarbons (THC) measurement using a gas analyzer (type MEXA-9100DEGR, Horiba). Exhaust in the central tank, which is used for the cell exposure experiments, is also used for measurements of PM mass, polycyclic aromatic hydrocarbons (PAH) measurements using Teflon coated glass fiber filters in combination with the adsorbent amberlite XAD-2 resin (Rohm and Haas, Sigma Aldrich; PAH analysis only), as well as elemental carbon/organic carbon (EC/OC) analysis (Shetty et al., 2006).

2.2. Calculation of diesel dose

The particle size distribution in the central tank was measured using a TSI Scanning Mobility Particle Sizer (SMPS, model 3936L22 TSI Incorporated, Shoreview, MN, USA). The particle number size distribution was used to calculate the mass concentration of the aerosol using the effective density of diesel soot (Maricq and Ning, 2004). The calculated mass concentration in the central tank was 0.206 ± 0.015 mg/m³ (n = 5). Delivered doses (DD), corresponding to the mass of particles applied to the inserts during the exposure time, was calculated as previously described (Zarcone et al., 2016), based on the SMPS measurements, the time of the exposure, the velocity flux and surface area of the inserts, using the following formula:

$$DD = \frac{f \times [PM] \times t}{SA}$$

In order to estimate the deposition efficiency of the particles in our system, five independent SMPS measurements of the first dilution of trade fuel emission were performed, to calculate the fractional efficiencies of particles based on the mass concentration (Maricq and Ning, 2004). The total deposition efficiency was thus estimated to be 1.9%. Deposited doses (*dd*), *i.e.* the actual mass of particles predicted to be deposited on the epithelial cell surface, were calculated based on the delivered doses and the deposition efficiency ($dd = DD \times 1.9\%$). PM concentrations, delivered doses and deposited doses for all exposure conditions are summarized in Table 1. The first dilution of the DE mixture produced during two independent sessions was used for the chemical characterization.

2.3. Cell culture

Primary bronchial epithelial cells (PBECs) were obtained from macroscopically normal lung tissue from patients undergoing resection surgery for lung cancer at the Leiden University Medical Center following the “Human Tissue and Medical Research: Code of Conduct for responsible use (2011)” guideline as previously described (Zarcone et al., 2016). Cells were used from 5 non-COPD and 7 COPD donors that were matched for age. COPD status was determined based on the patient's lung function following the Chronic Obstructive Lung Disease (GOLD) classification (Vestbo et al., 2013): one GOLD III, five GOLD II and one GOLD I. Lung function data are shown in Table 2.

Following expansion, well-differentiated cultures were obtained by seeding passage 2 PBECs in 12 well-plate Transwell inserts (Corning Costar Corporation, Cambridge, MA) and feeding these every two days

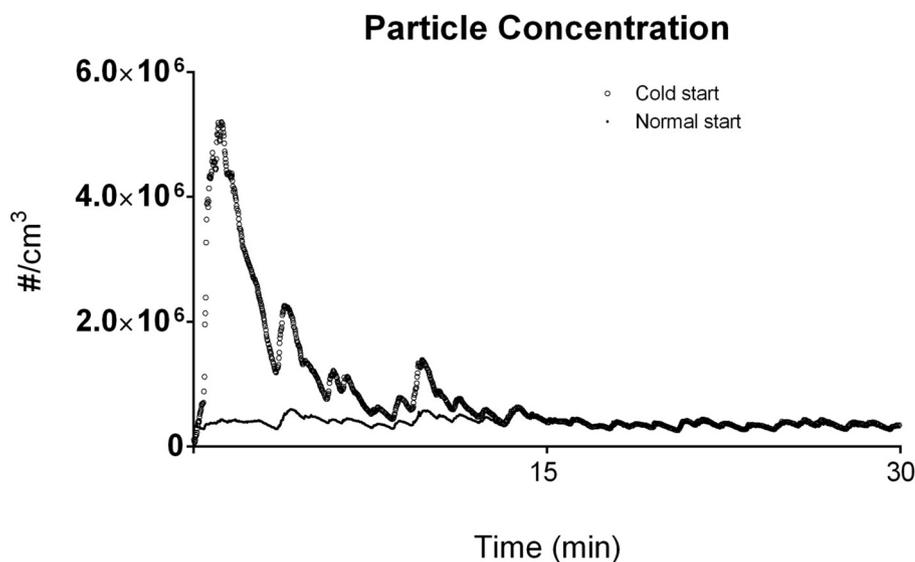


Fig. 1. Particle concentrations during cold start and normal start exposures.

Particle concentration ($\#/cm^3$) obtained by SMPS measurements of the DE emissions collected during the first 30 min cycle (“cold start”) and a second cycle (“normal start”) from an engine that was prewarmed by running for 30 min.

Table 1
PM dose delivered to ALI-PBEC cultures during different exposure times.

Condition	<i>In vitro</i> exposure time (min)	PM (mg/ m^3) ^a	Delivered dose (DD, $\mu g/cm^2$) ^b	Deposited dose (dd, ng/cm^2) ^b
High	60	0.206	0.055	1.060
High	150	0.206	0.138	2.650
Low	360	0.034	0.055	1.060
Mid	360	0.082	0.132	2.544
High	360	0.206	0.331	6.359

^a PM concentrations were calculated based on SMPS measurements in the mixture collected in the central tank (17-fold diluted). Low and mid concentrations are calculated from the high concentration, using a dilution factor of 2.5 and 6, respectively.

^b Delivered dose and deposited dose were calculated as described in the Methods section based on a flow of 5 mL/min, and a surface area of 1.12 cm^2 .

Table 2
Donor characterization.

Characteristics	Non-COPD (n = 5)	COPD (n = 7)
Age (yr)	65 ± 6	69 ± 7
Gender (male/female)	3/2 (M/F)	4/3 (M/F)
FEV ₁ (% pred)	90.82 ± 19.4	70 ± 19*
Pre-BD FEV ₁ (L)	2.9 ± 0.8	1.9 ± 0.5*
FVC (% pred)	95.0 ± 14.3	93.7 ± 21.1
FVC (L)	3.7 ± 0.8	3.3 ± 0.9
FEV ₁ /FVC (%)	76.5 ± 4.9	59.3 ± 5.9*
GOLD stage I/II/III	–	1/5/1

Data are mean ± SD.

Abbreviation: FEV₁: forced respiratory volume 1; Pre-BD: precedent the use of bronchodilator; FVC: forced vital capacity; GOLD: Chronic Obstructive Lung Disease.

* $p < 0.01$ vs non-COPD subjects by Mann-Whitney *t*-test.

with a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and bronchial epithelial cell growth media (BEGM, Lonza, Verviers, Belgium) supplemented with penicillin/streptomycin (Lonza), BEGM SingleQuot (Lonza), bovine serum albumin (BSA, Sigma, St. Louis, USA) and retinoic acid (Lonza) and culture at 37 °C with 5% CO₂ (Zarcone et al., 2016). After reaching confluence, apical medium was removed and cells were cultured for two weeks at the air-liquid interface (ALI) to allow mucociliary differentiation (Zarcone et al., 2017; Zuyderduyn et al., 2011).

2.4. UV-inactivated non-typeable *Haemophilus influenzae*

A log-phase culture of non-typeable *Haemophilus influenzae* (NTHi)

strain D1 (Groeneveld et al., 1990) was obtained from a single colony as previously described (Zarcone et al., 2017). After reaching the log-phase, bacterial cells were washed and diluted in phosphate-buffered saline (PBS) to a concentration of 1×10^9 colony forming unit (CFU)/mL. Bacteria were killed by exposure to UV-light for 2 h.

2.5. Cell treatments and controls

Selection of exposure duration and incubation time with NTHi were based on observations obtained during our previous studies (Zarcone et al., 2016; Zarcone et al., 2017). Following DE exposure, inserts were transferred into new 12 well-plates with fresh media. In the experimental session investigating prolonged diesel exposure without subsequent bacterial treatment, cells were incubated with basal media only. Cell incubation with bacteria was performed following exposure to DE or air for various durations, followed by apical addition of 100 μL of 10^9 CFU/mL of NTHi in PBS or PBS alone as controls (Table 3). For all experiments, cells from each donor were incubated also without placement in the exposure unit as unexposed controls (U). Furthermore, these unexposed cells were also treated with 20 ng/mL of transforming growth factor-beta (TGF β) in the basal media (oxidative stress positive control), 100 μL of 10^9 CFU/mL of NTHi apically (NTHi positive control), or 100 μL of 0.1% Triton- \times 100 apically (cytotoxicity positive control).

After exposure, apical PBS with or without NTHi was collected to perform subsequent analysis. Apical washes with 100 μL of PBS were collected for inserts which were incubated without PBS apically.

2.6. Transepithelial electrical resistance (TEER) and LDH release

Electrical resistance and lactate dehydrogenase (LDH) release were measured as previously described (Zarcone et al., 2016) and express respectively as $\Omega \cdot cm^2$ and as % of LDH release in 0.01% Triton X-100 (Sigma)-treated positive control.

2.7. Quantitative real-time polymerase chain reaction (rtPCR)

After collection of apical washes and basal media, cell lysates were obtained by adding 200 μL of lysis buffer (Promega, Leiden, NL) and total RNA extraction was extracted using the Maxwell[®] 16 simplyRNA Tissue Kit (Promega). Next, complementary DNA (cDNA) was obtained using moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega), as previously described (Zarcone et al., 2016). Quantitative rtPCR reactions were performed using iQSybr green Supermix (Biorad)

Table 3
Overview of exposure conditions and treatment.

	Condition	Engine start	Exposure (h)	Incubation (h)	Apical PBS	Apical PBS + NTHi	non-COPD (n)	COPD (n)	
Fig. 2	Air	Warm	6	1:30	17:00	–	–	5	–
	Low DE	Warm	6	1:30	17:00	–	–	5	–
	Mid DE	Warm	6	1:30	17:00	–	–	5	–
	High DE	Warm	6	1:30	17:00	–	–	5	–
Figs. 3, 4, 5	Air	Warm	6	3:00	17:00	+	–	3	–
	High DE	Warm	6	3:00	17:00	+	–	3	–
	Air	Warm	6	3:00	17:00	–	+	3	–
	High DE	Warm	6	3:00	17:00	–	+	3	–
Fig. 6	Air	Warm	2:30	3:00	+	–	5	7	
	High DE	Warm	2:30	3:00	+	–	5	7	
	Air	Warm	2:30	3:00	–	+	5	7	
	High DE	Warm	2:30	3:00	–	+	5	7	
Fig. 7	Air	Cold	1	3:00	+	–	5	–	
	High DE	Cold	1	3:00	+	–	5	–	
	Air	Cold	1	3:00	–	+	5	–	
	High DE	Cold	1	3:00	–	+	5	–	
	Air	Warm	1	3:00	+	–	2	–	
	High DE	Warm	1	3:00	+	–	2	–	
	Air	Warm	1	3:00	–	+	2	–	
	High DE	Warm	1	3:00	–	+	2	–	

Table 4
Primers sequences for quantitative qPCR.

Gene	Tm (°C)	Forward sequence/reverse sequence	GeneBank accession no. or reference
<i>ATP5B</i>	63°	TCACCCAGGCTGGTTCAGA AGTGGCCAGGTTAGGCTGAT	NM_001686
<i>RPL13A</i>	63°	AAGGTGGTGGTCGTACGCTGTG CGGGAAGGTTGGTTCATCC	NM_012423
<i>HMOX1</i>	63°	AACCCCTGAACAACGTAGTCTGCGA ATGGTCAACAGCGTGGACACAAA	NM_002133
<i>HSPA5/BiP</i>	62°	CGAGGAGGAGCAAGAAGG CACCTTGAACGGCAAGAAGT	NM_001025433
<i>DDIT3/ CHOP</i>	62°	GCACCTCCAGAGCCCTCACTCTCC GTCTACTCCAAGCCTTCCCTGCGG	NM_001195053.1
<i>PPP1R15A/ GAD- D34</i>	62°	ATGTATGGTGAGCGAGAGGC GCAGTGTCTTATCAGAAGGC	(Oh-Hashi et al., 2001)
<i>XBP1 spl</i>	62°	TGCTGAGTCCGAGCAGGTG GCTGGCAGGCTCTGGGGAAG	(van Schadewijk et al., 2012)
<i>CXCL8</i>	59°	CTG GAC CCC AAG GAA AAC TGG CAA CCC TAC AAC AGA C	NM_000584
<i>DEFB4A/ hBD2</i>	62°	ATCAGCCATGAGGGTCTTG GCAGCATTTTGTCCAGG	NM_004942

and the CFX-384 rtPCR system (Bio–Rad Laboratories, Veenendaal, The Netherlands) as previously described (Zarcone et al., 2016). The standard curve method was applied to obtain arbitrary gene expression. Primers for the various target genes are listed in Table 4. Expression of markers of interested was normalized on the expression of ATP synthase subunit beta (*ATP5b*) and ribosomal protein L13A (*RPL13a*), selected via the GeNorm method (Vandesompele et al., 2002). *HMOX1* and *NQO1* mRNA were selected as marker of oxidative stress response; the ISR to the unfolded protein response (UPR) were investigated via the analyses of *CHOP*, *PPP1R15A/GADD34*, *XBP1 spl* and *HSPA5/ BiP* mRNA expression. Finally innate immune response was studied following the expression of *CXCL8* mRNA and of the antimicrobial peptide *DEFB4A* mRNA.

2.8. Statistical analysis

For both non-COPD and COPD groups, the effects of DE exposure were compared to air controls with a one-way ANOVA with a Games-Howell post-hoc test, since the assumption of the equality variances was not met. To investigate the significance of interactions between DE and

NTHi, two-way ANOVA statistical analyses were performed for samples exposed to DE and treated with NTHi. Three-way ANOVA analysis was used to highlight potential significant interactions between disease conditions, DE exposure, NTHi exposure, or between cold/warm start, DE exposure and NTHi exposure. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

We first investigated the effect of prolonged exposures (up to 6 h) to three DE doses. Secondly, we studied the influence of diesel exposure on the cellular response to treatment with NTHi following *i*) prolonged diesel exposure (6 h) of non-COPD donors; *ii*) 2:30 h diesel exposure comparing non-COPD and COPD cells; and *iii*) 1 h diesel exposure mimicking cold start conditions. Exposure conditions are described in Table 3. Chemical characterization of the diesel exhaust was performed at the first dilution in the CVS tunnel and shown in Table 5.

3.1. Six hours exposure to diesel exhaust

To investigate the dynamics of the epithelial response to 6 h exposure to DE (low, mid and high) or air, the oxidative stress response was investigated after short (1:30 h) and longer (17 h) post-exposure incubation times. DE exposure caused a dose-dependent *HMOX1* and *NQO1* mRNA increase at 1:30 h post-exposure, which was statistically significant in the high and mid DE-exposed cells. *HMOX1* and *NQO1* expression at 1:30 h post-exposure was significantly increased in high DE-exposed cells compared to air- and low DE-exposed cells (*HMOX1*: $p = 0.042$ and $p = 0.044$ respectively; *NQO1*: $p = 0.028$ and $p = 0.023$, Fig. 2A and C). In addition, *HMOX1* and *NQO1* expression was also significantly increased in the mid DE exposed cells compared to air-exposed control cells (*HMOX1* $p = 0.002$ and $p = 0.001$ respectively, Fig. 2A and C). *NQO1* was also significantly higher after exposure to mid DE compared to low DE-exposed cells ($p = 0.019$, Fig. 2C). At 17 h post-exposure, the increase in *HMOX1* (Fig. 2B) and *NQO1* (Fig. 2D) mRNA did not reach statistical significance. Six hours DE exposure did not affect epithelial barrier function or cause cytotoxicity (Supplementary Fig. 1A–D). In TGF β -treated cultures, *HMOX1* induction occurred only at 17 h, whereas *NQO1* was not increased (Supplementary Fig. 2A and B). No effect on markers of the integrated stress response or inflammatory response was observed (Supplementary Fig. 3).

Table 5
Diesel exhaust chemical characterization.

Fueltype	Diesel CS (average)	Standard deviation	Diesel (average)	Standard deviation
SMPS (#/cm ³)	1.2 * 10 ⁶	^a	0.83 * 10 ⁶	± 0.24 * 10 ⁶
PM ₁₀ (µg/m ³)	456	^a	295	^a
OC (µg/m ³)	151	^a	66	^a
EC (µg/m ³)	119	^a	136	± 23
Hopanes/steranes (ng/m ³)	18.21	^a	11.02	± 2.77
sumPAH total (ng/m ³)	4350	^a	2259	± 39
nitro PAH (ng/m ³)	4.40	^a	2.38	^a
oxy PAH (ng/m ³)	129	^a	105	^a
nmol DTT/min/m ³	-0.22	^a	-0.10	^a
Metals (ng/m ³)	4015	^a	3206	± 801
CO ₂ (volume %)	0.38	± 0.00	0.36	± 0.00
CO (ppm)	12.86	± 0.58	4.92	± 0.31
NO _x (ppm)	40.98	± 0.45	31.60	± 1.29
THC (ppm)	5.02	± 0.23	3.89	± 0.26

Characterization of the first dilution of the DE mixtures (high diesel dose) produced by a Euro V bus engine in a cold start set up (Diesel CS) and after stabilization of the emission (Diesel). For each set up, measurements were performed on the emission produced in two independent sessions. Abbreviations: SMPS: scanning mobility particle sizer; PM: particulate matter; OC: organic carbon; EC: elemental carbon; PAH: polycyclic aromatic hydrocarbon; DTT: dithiothreitol; CO₂: carbon dioxide; CO: carbon monoxide; NO_x: nitrogen oxide; THC: total hydrocarbon.

^a Only one measurement.

3.2. Effects of 6 h of diesel exhaust exposure on the response to NTHi

To assess the effect of DE exposure on subsequent microbial exposure to NTHi, cells were first exposed for 6 h to DE (high) or air, before addition of NTHi. No effects of these treatments on barrier function (TEER) or cytotoxicity (LDH release) were noted (Supplementary fig. 4). At 3 h, but not at 17 h after DE exposure, there was a trend towards higher expression of *HMOX1* and *NQO1* only in cells exposed to both DE and NTHi, but this did not reach statistical significance (Fig. 3A, B, C and D); NTHi alone also did not affect these markers in non-exposed cells treated with bacteria alone (Supplementary fig. 5).

Analyses with a two-way ANOVA demonstrated significant interactions between DE and NTHi only in the induction of *PPP1R15A/GADD34* mRNA ($p = 0.011$), showing that DE enhanced the NTHi-induced expression of *PPP1R15A/GADD34* (Fig. 4A). The combination of DE exposure and NTHi treatment increased *PPP1R15A/GADD34* mRNA expression at 3 h after DE exposure compared to the air-exposed and NTHi-treated cells (Fig. 4A), whereas no effect on *CHOP* mRNA induction was observed (Supplementary fig. 5A and B). Moreover, whereas *PPP1R15A/GADD34* mRNA was increased, DE caused a significant inhibition of the NTHi-induced expression of the endoplasmic reticulum chaperone HSPA5/BiP ($p = 0.026$, Fig. 4C). No further induction was observed for these two markers of the unfolded protein response (UPR) at later time points (Fig. 4B and D). *XBPI spl* was induced only at a later time point in NTHi-treated cells, with a further but non-significant increase in DE-exposed cells incubated with or without NTHi (Fig. 4E and F). NTHi alone induced a non-significant increase in *PPP1R15A/GADD34* mRNA expression at 3 h incubation in cells that were not exposed to DE (Supplementary figs. 5 and 7).

The combination of DE and NTHi treatment caused a non-significant but marked increase in the inflammatory response, as shown by the

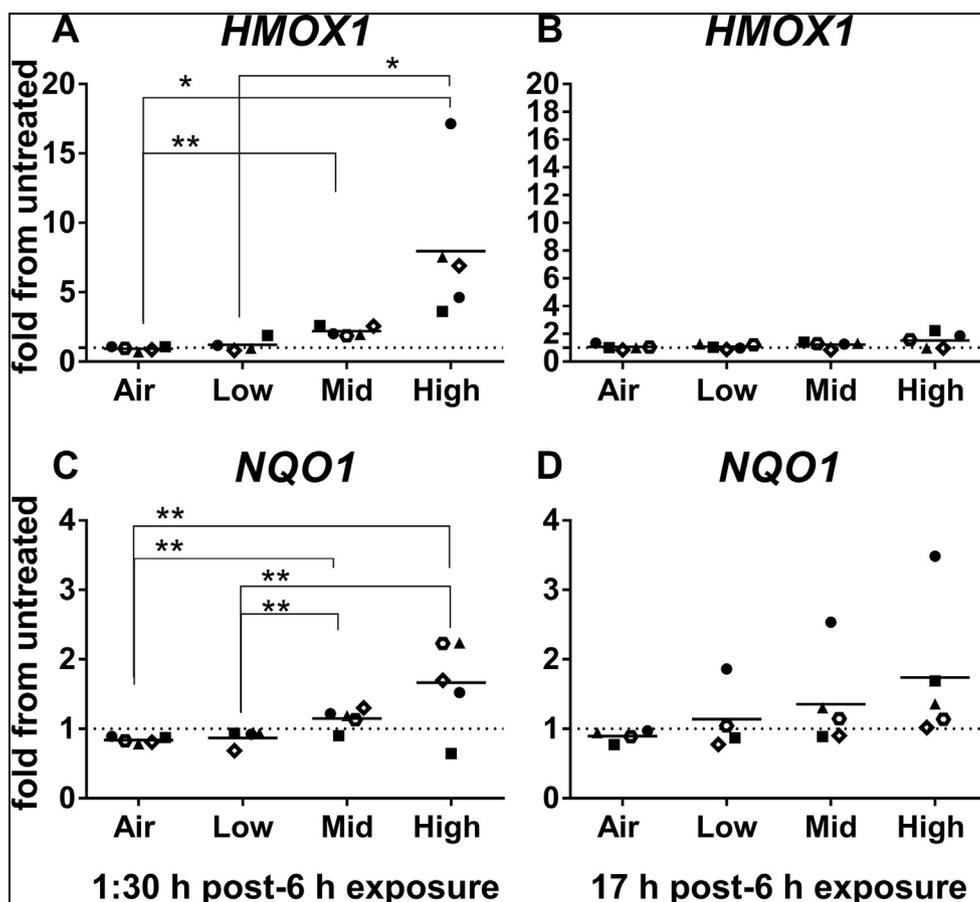


Fig. 2. Oxidative stress response to prolonged exposure to diesel exhaust.

Induction of *HMOX1* (Fig. 2A and B) and *NQO1* (Fig. 2C and D) in cells from 5 non-COPD donors exposed for 6 h to air or diesel exhaust (low, mid and high) and then further incubated for 1:30 (Fig. 2A and C) or 17 h (Fig. 2B and D). Data are represented as single values, with the mean as horizontal bars, of the fold increase from untreated control (dashed line) after normalization on two reference genes, *ATP5b* and *RPL13a*. Differences were considered statistically significant at * $p < 0.05$ and ** $p < 0.01$ with One-way ANOVA with Games-Howell post-hoc test.

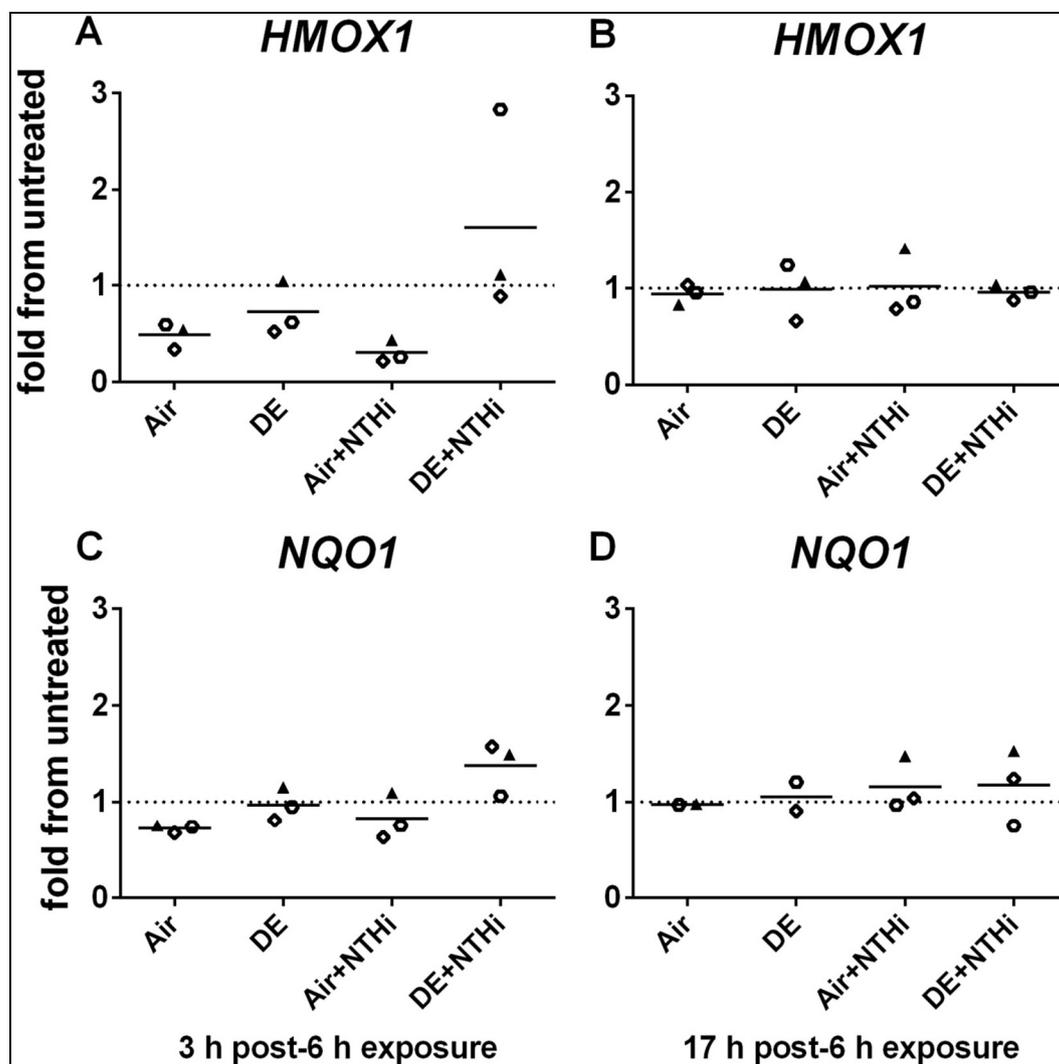


Fig. 3. Oxidative stress response to prolonged DE exposure followed by NTHi treatment.

Cells from 3 non-COPD donors were exposed for 6 h to air or diesel exhaust (high) and then incubated for 3 (Fig. 3A and C) or 17 h (Fig. 3B and D) with or without NTHi on the apical side. Data are represented as single values and the means are indicated by horizontal bars. *HMOX1* (Fig. 3A and B) and *NQO1* (Fig. 3C and D) mRNA expression is presented as fold increase from untreated control (dashed line) after normalization on two reference genes, *ATP5b* and *RPL13a*.

mRNA induction of *CXCL8* at 3 h incubation time (Fig. 5A). However, DE showed a tendency to decrease the NTHi-induced increase of *DEFB4A* mRNA at 17 but not 3 h post-DE (Fig. 5C and D).

3.3. Comparison of the response to diesel exhaust and NTHi of COPD and non-COPD cultures

Next we investigated whether ALI-PBEC cultures derived from COPD or non-COPD patients demonstrated a different response to diesel exhaust and NTHi. No effect of these exposures on TEER or cytotoxicity was observed in both groups (data not shown). DE induced *HMOX1* mRNA expression in cells from both non-COPD and COPD donors ($p = 0.021$ and $p < 0.000$, Fig. 6A). In the DE-exposed COPD cells, NTHi significantly reduced the DE-induced *HMOX1* expression ($p = 0.001$). This effect was further confirmed by the results of a two-way ANOVA analysis, which showed a significant interaction between DE and NTHi in the modulation of *HMOX1* in the COPD group ($p < 0.000$). NTHi caused an increase in *PPP1R15A/GADD34* mRNA that reached significance in the COPD donors, both in cells previously exposed to air or DE ($p = 0.011$ and $p = 0.039$; Fig. 6B). NTHi caused an increase in *CXCL8* mRNA that reached significance in the COPD donors, and that was not further increased by prior DE exposure ($p < 0.001$ [NTHi-air] and $p = 0.001$ [NTHi-DE]; Fig. 6C). DE

exposure caused an apparent inhibition of the NTHi-induced *DEFB4A* expression in cell cultures from both patient groups (Fig. 6D). However, as for *HMOX1*, this interaction between DE and NTHi in the modulation of *DEFB4A* expression was only significant in cells from COPD patients ($p = 0.009$; two-way ANOVA analysis). mRNA induction of *DEFB4A*, (in all both groups) and *CXCL-8* (in non-COPD donors) was more marked in the air-exposed cells (exposure modules) than in the incubator controls (supplementary fig. 6). Three-way ANOVA analyses indicated a lack of significant interaction between disease status, DE exposure and NTHi treatment for all markers studied.

3.4. Effect of short cold start exposure on the cellular response to NTHi

Compared to non-COPD cells exposed to already stabilized emissions ($n = 2$) (Fig. 1), no differences in response were observed in cells exposed in a cold start set up (Fig. 7, Supplementary table 2). 1 h DE-cold start exposure caused a small induction of *NQO1* mRNA without reaching statistical significance (Fig. 7A). NTHi treatment in exposed cells appeared to cause a higher increase in *NQO1* mRNA than DE in both groups (Fig. 7A), while *NQO1* expression was not induced by NTHi in incubator controls (Supplementary fig. 7). *HMOX1* mRNA induction was not detected (data not shown). Treatment with NTHi alone (no DE exposure) induced *CXCL8* mRNA (Fig. 7C). *CXCL8* was also

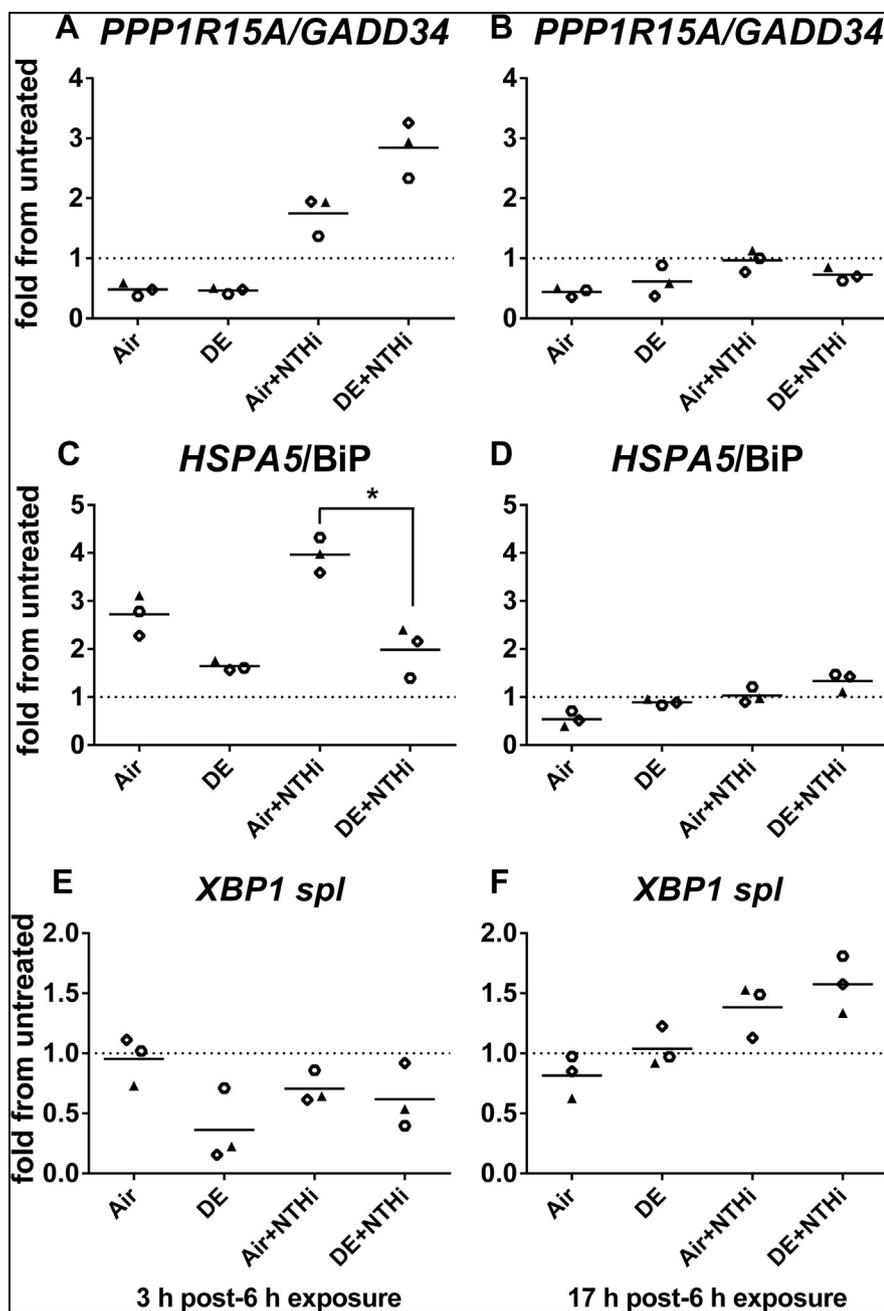


Fig. 4. Expression of markers of the Unfolded Protein Response and Integrated Stress Response after 6 h DE exposure followed by NTHi treatment.

PPP1R15A/GADD34 (Fig. 4A and B), *BiP* (Fig. 3C and D) and *XBP1 spl* (Fig. 4E and F) mRNA expression (unfolded protein response) in cells from 3 non-COPD donors exposed for 6 h to air or diesel exhaust (high) and then incubated for 3 (Fig. 4A, C and E) or 17 h (Fig. 4B, D and F) with or without NTHi on the apical side. Data are expressed as single values and the mean is indicated by horizontal bars. Gene expression is presented as fold increase from untreated controls (dashed line) after normalization using two reference genes, *ATP5b* and *RPL13a*. Differences were considered statistically significant at $*p < 0.05$ with One-way ANOVA with Games-Howell post-hoc test. Furthermore, a significant interaction between the effect of DE and NTHi exposure on the induction of *PPP1R15A/GADD34* mRNA was present ($p = 0.011$, two-way ANOVA).

significantly increased by NTHi after DE exposure using either a cold or a warm engine ($p = 0.008$ and $p = 0.019$, respectively, Fig. 7C). *PPP1R15A/GADD34* mRNA appeared somewhat higher in cells exposed to cold start DE and incubated with NTHi compared to NTHi or DE alone. Importantly, 1 h exposure to DE in both exposures already caused a non-significant inhibition of *DEFB4A* mRNA induction by NTHi (Fig. 7D). In non-exposed control cells treated with NTHi, the increase in *PPP1R15A/GADD34* and *DEFB4A* mRNA was similar as in the air-exposed cells also treated with NTHi, while NTHi-induced *CXCL8* mRNA induction was higher in the air-exposed cells than the non-exposed controls (Supplementary fig. 7). Three-way ANOVA analyses indicated a lack of significant interaction between engine starting condition, DE exposure and NTHi treatment for all markers studied.

4. Discussion

Our data obtained using a Euro V bus engine emission showed a

diesel exhaust (DE)-mediated inhibition of the NTHi-induced expression of the *DEFB4A* (antimicrobial peptide) mRNA in primary bronchial epithelial cells (PBEC) differentiated at the air liquid interface (ALI). Inflammatory (*CXCL8*) and integrated stress (*PPP1R15A/GADD34*) responses were activated in cells exposed to DE and treated with NTHi, while DE caused an inhibition of the NTHi-induced expression of the chaperone protein BiP (*HSPA5*). The DE dose-dependent oxidative stress response was detected shortly after exposure to the higher diesel concentrations. Interestingly, while increases in most mediators required prolonged exposures to DE, the decrease in NTHi-induced *DEFB4A* was already observed after 1 h exposure.

We previously described the inhibitory effects of DE on the antimicrobial response of human bronchial epithelial cells exposed to DE using a diesel-fueled generator at steady load state conditions (Zarcone et al., 2017). Using a delivered dose (DD) of $0.404 \mu\text{g}/\text{cm}^2$, the effects on the inhibition of the NTHi-induced *S100A7* and *DEFB4A* mRNA expression occurred in parallel with a pronounced oxidative stress

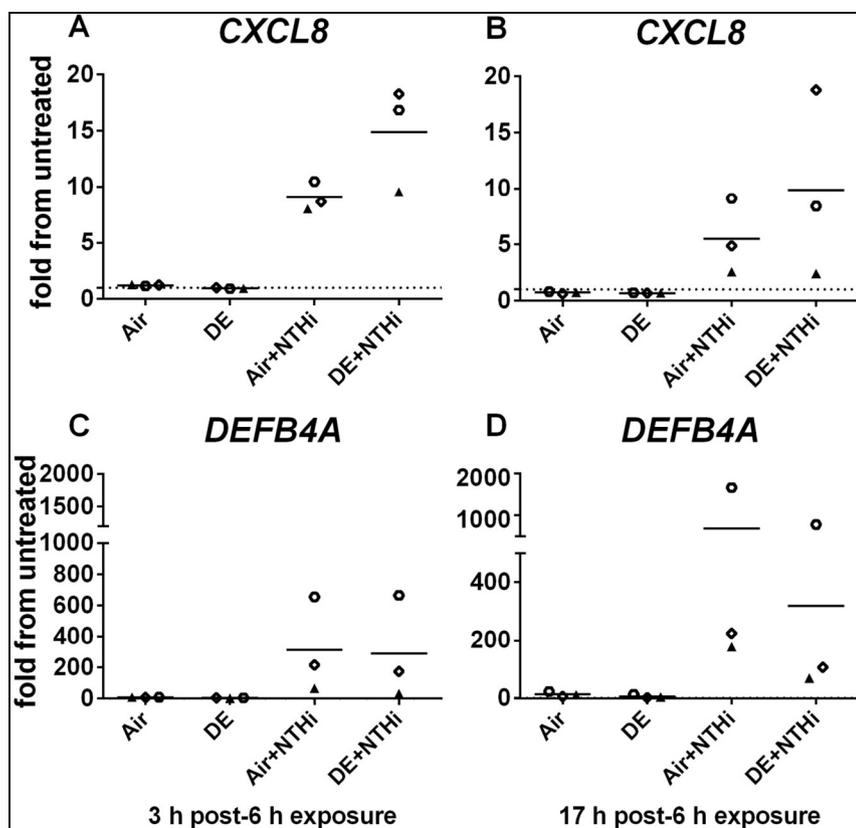


Fig. 5. Inflammatory and antimicrobial response to 6 h DE exposure followed by NTHi treatment.

CXCL8 (inflammatory response marker) and *DEFB4A* (encoding for antimicrobial peptide hBD2) mRNA expression in cells from 3 non-COPD donors exposed for 6 h to air or diesel exhaust (high) and then incubated for 1:30 (Fig. 5A and C) or 17 h (Fig. 5B and D) with or without NTHi on the apical side. Data are represented as single values, with the mean as horizontal bars, of the fold increase from untreated control (dashed line) after normalization on two reference genes, *ATP5b* and *RPL13a*.

response, as shown by *HMOX1* expression (Zarcone et al., 2017). Here, we demonstrate that DE also reduces expression of the antimicrobial peptide *DEFB4A*, but not of *S100A7* (data not shown), in NTHi-treated ALI-PBECs using DE containing ~3 times lower emission levels (PM mass based) produced by a Euro V bus engine (DD = 0.138 $\mu\text{g}/\text{cm}^2$) and still present with lower doses (DD = 0.055 $\mu\text{g}/\text{cm}^2$). However, the significant DE dose-dependent oxidative stress response was markedly lower after prolonged exposure (6 h, 0.331 $\mu\text{g}/\text{cm}^2$), compared to previous observations in which we delivered ~0.375 $\mu\text{g}/\text{cm}^2$ of DE particles in 1 h exposure (Zarcone et al., 2016; Zarcone et al., 2017). The transcription factors Nrf2 and NF- κ B have been suggested to mediate a transient induction of *HMOX1* mRNA (Pronk et al., 2014). This transient increase is also clearly illustrated following 6 h DE exposure, when an increase was observed at 1:30 h post-exposure not at 17 h post-exposure. Furthermore, *HMOX1* expression was further reduced following shorter exposures to lower doses of DE particles, suggesting a close relation with both level of DE exposure and timing after the exposure. Interestingly, the DE-mediated inhibition of *DEFB4A* mRNA expression was observed after all exposure durations, also in absence of *HMOX1* mRNA induction. Notably, the large variation in *DEFB4A* induction among donors reduced the power to detect significant effects in small groups.

NTHi-treatment of DE-exposed cells increased the expression of the neutrophil chemoattractant *CXCL8* using DD of 0.138 and 0.331 $\mu\text{g}/\text{cm}^2$ of diesel exhaust particles. Neutrophilia is a common feature in the lungs of COPD patients (Brusselle et al., 2011). In a previous study, we showed that DE alone induced *CXCL8* expression (Zarcone et al., 2016), which was not observed here using lower diesel levels (based on PM mass). Diesel exhaust enhanced the NTHi-induced inflammatory response only in non-COPD donors without reaching statistical significance. Direct oxidative stress or extended protein damage can both increase *PPP1R15A/GADD34* expression as a result of activation of the unfolded protein response (UPR) or the integrated stress response (ISR) (Ron and Walter, 2007; van 't Wout et al., 2014). DE exposure alone did

not induce activation of *PPP1R15A/GADD34* or other UPR markers. In contrast, we previously noted that DE that was generated by an off-road engine did increase expression of the ISR markers *GADD34* and *CHOP* (Zarcone et al., 2016). This difference is most likely explained by the different composition of DE generated by the two engines. In contrast, after prolonged exposure to Euro V diesel emissions (DD = 0.331 $\mu\text{g}/\text{cm}^2$), the presence of bacteria increased *PPP1R15A/GADD34* expression, indicating an interaction between diesel and bacterial exposures. *PPP1R15A/GADD34* has been suggested to be a pro-apoptotic marker (Tabas and Ron, 2011), especially in chronic disease conditions such as COPD. Experiments using chronic exposure to diesel might better address the role of *PPP1R15A/GADD34* in the response of primary bronchial epithelial cells. Protein folding can be restored by several chaperone proteins. Among them, *HSPA5/BiP* is induced alongside with *PPP1R15A/GADD34* and the transcriptional factor spliced *XBP1* during the unfolded protein response (UPR) to endoplasmic reticulum (ER) stress (Ron and Walter, 2007). We observed that DE exposure decreased the ability of NTHi to increase expression of *HSPA5/BiP*, suggesting partial impairment of the unfolded protein response. The functional relevance of this impairment requires further study, but may involve a limitation in the rescue of misfolded/damage proteins. Collectively, these data suggest a synergist effect of diesel and NTHi exposure in the activation of the inflammatory and integrated stress response in parallel with a reduction of chaperone mRNA transcription. Additional studies are necessary to confirm the potential implication of these findings, especially in relation to COPD exacerbations, which are commonly associated with respiratory tract infections. Furthermore, since *HMOX1* and integrated stress markers are rapidly induced after DE exposure (Zarcone et al., 2016), time of exposure might influence the detection of markers even at the same incubation time points.

Use of primary cells allows the direct comparison of cells from different donors in controlled *in vitro* conditions (Zarcone et al., 2017). Here, we compared the response of cells from non-COPD and COPD donors to the presence of bacteria after exposure to DE. 2:30 h exposure

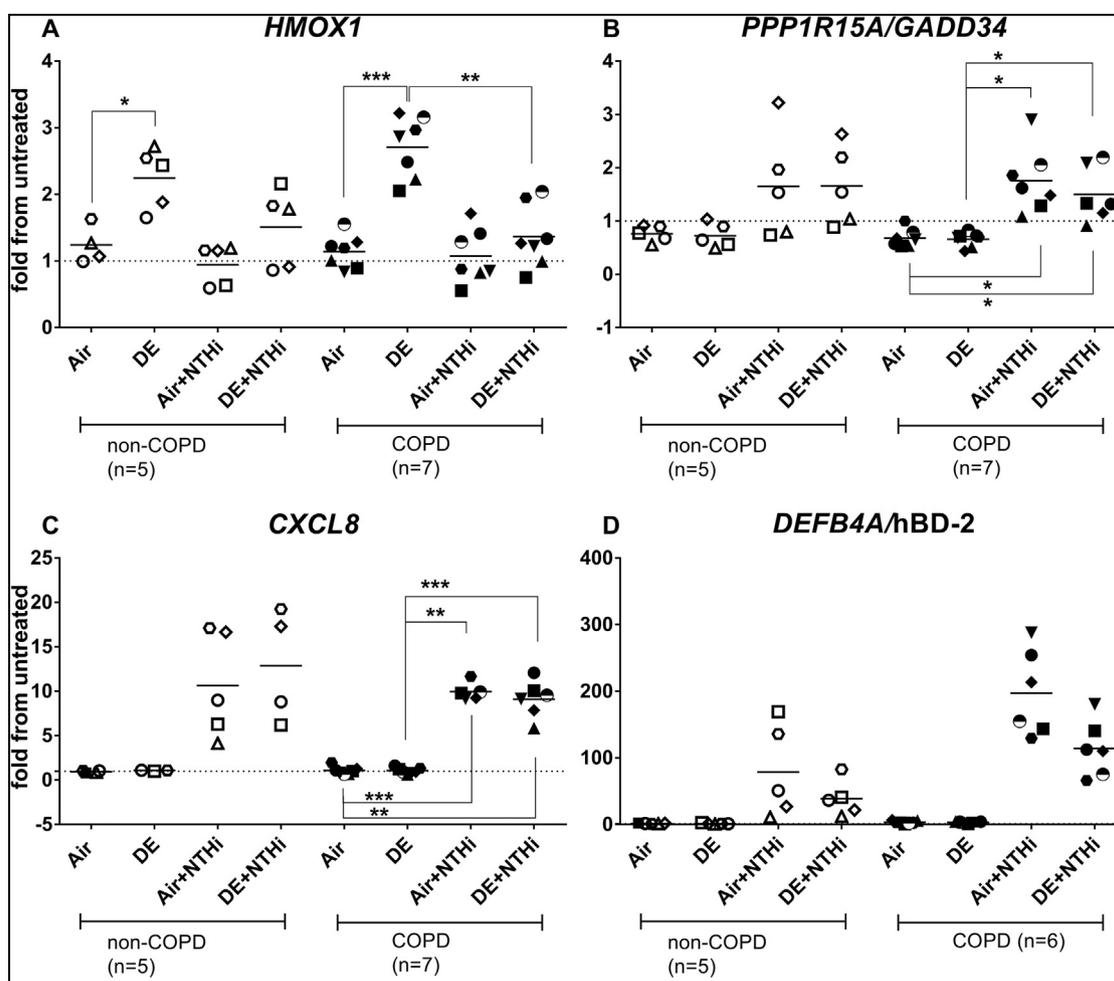


Fig. 6. Comparison of the response of COPD and non-COPD cultures to DE exposure followed by NTHi treatment.

Cells from 5 non-COPD and 7 COPD donors were exposed for 2:30 h to air or diesel exhaust (high) and then incubated for 3 h with or without NTHi on the apical side. Data are represented as single values, with the mean as horizontal bars, of the fold increase from untreated control (dashed line). Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ with One-way ANOVA with Games-Howell as post-hoc test. *HMOX1* (Fig. 6A), *PPP1R15A/GADD34* (Fig. 6B), *CXCL8* (Fig. 6C) and *DEFB4A* (Fig. 6D) mRNA induction is expressed as fold increase from untreated control (dashed line) after normalization on two reference genes, *ATP5b* and *RPL13a*. For *DEFB4A* a significant outlier was excluded (Fig. 6D). Exposures to DE and NTHi demonstrated a significant interaction in the modulation of *HMOX1* ($p < 0.001$) and *DEFB4A* mRNA ($p = 0.009$) only in the COPD group (two-way ANOVA analysis).

to Euro V diesel exhaust ($DD = 0.138 \mu\text{g}/\text{cm}^2$) resulted in an increased oxidative stress response which was significant in cells from COPD and non-COPD control donors. COPD donors showed a higher level of *HMOX1* expression upon DE exposure compared to non-COPD donors, but the difference was not significant likely reflecting the difference in sample size between groups. Nevertheless, this result is in line with *in vivo* observations, which suggest a higher susceptibility to oxidative stress of COPD patients exposed to diesel exhaust (Ling and van Eeden, 2009; Ni et al., 2015). DE caused a significant inhibition of *DEFB4A* mRNA in cultures from COPD patients, whereas this inhibition did not reach statistical significance in cultures from non-COPD patients. This inhibition may increase susceptibility to respiratory pathogens such as non-typeable *Haemophilus influenzae* (NTHi).

In order to gain further insight into the effect of diesel emissions, cells from non-COPD donors were also exposed to emissions from a Euro V bus engine generated during a cold engine start. No differences were observed in the response of cells exposed for 1 h to stabilized emission compared to cells exposed to diesel emission derived from a cold engine start characterized by an initial peak of diesel particles concentration. The difference in the number of donors of the two groups might have limited the possibility to observe significant differences. Importantly, 1 h exposure to diesel ($DD = 0.055 \mu\text{g}/\text{cm}^2$) was sufficient to reduce the NTHi-induced *DEFB4A* and activated the

inflammatory and integrated stress response in presence of bacteria but not *HMOX1*. In an alternative model of exposure to CUO, differences in donor responses constitute one of the sources of variation in nanoparticles studies (Kooter et al., 2017). Indeed, due to the large donor variation in response to cold and normal start exposure, we are not able to draw conclusions about the comparison of the two exposure scenarios.

The realistic exposure conditions generated at TNO's Automotive Powertrain test center allowed us to investigate the response of state-of-the-art airway epithelial cell cultures to whole diesel exhaust and bacteria. However, a clear limitation of our study is that only cells of a relatively small number of donors could be evaluated, and that the COPD patients did not have advanced disease. Nevertheless, despite the limitations inherent to any *in vitro* approach, these studies provide important additional information on the possible adverse health effects resulting from exposure to modern traffic.

5. Conclusion

Our results show that emissions generated by a city bus with Euro V diesel engine may cause adverse effects on the human airway epithelium, including activation of oxidative stress responses and inhibition of antimicrobial peptide expression. Furthermore, our study indicates that

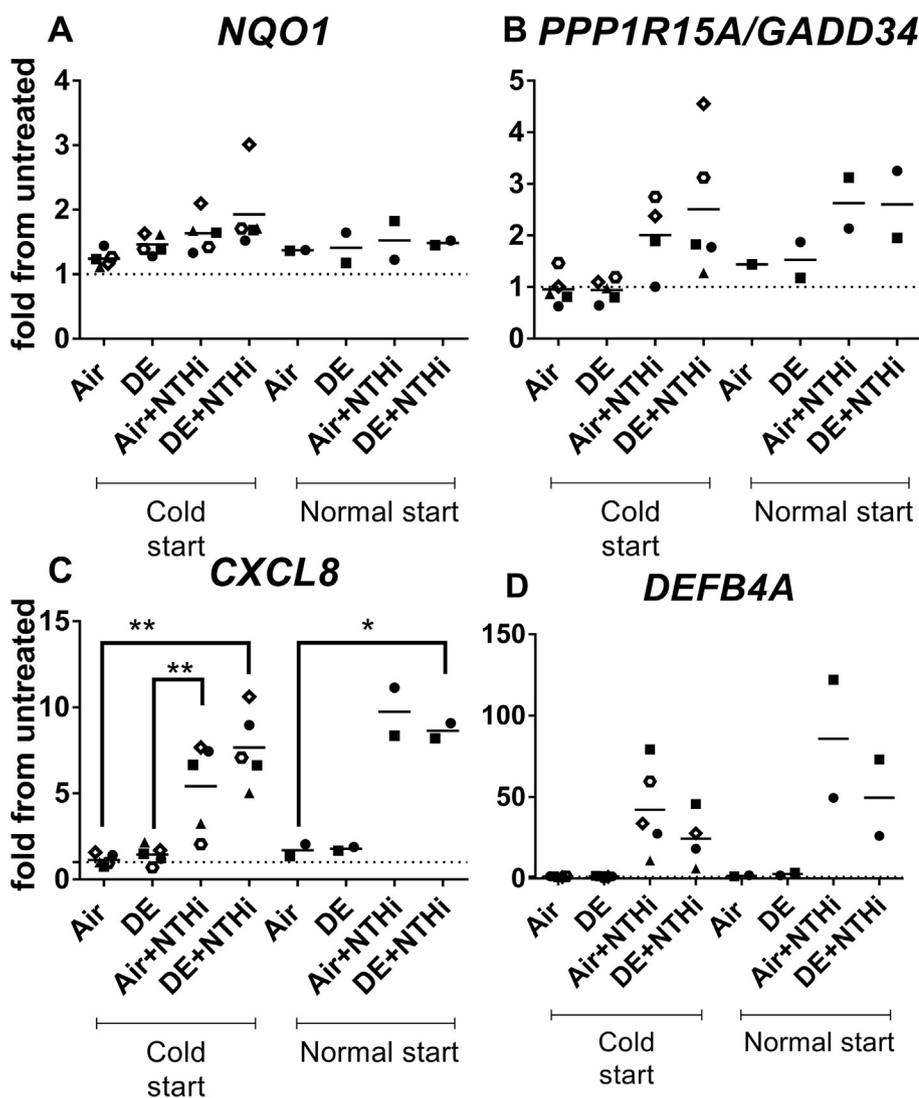


Fig. 7. Effect of DE generated by a short cold engine start on the cellular response to NTHi.

Cells from 5 non-COPD donors were exposed for 1 h to air or diesel exhaust (high) with a cold start exposure set up and then incubated for 3 h with or without NTHi on the apical side in two independent exposure sessions. Data are expressed as single values of the mean for each donor, while the mean for exposure conditions is indicated by horizontal bars. *NQO1* (Fig. 7A), *PPP1R15A/GADD34* (Fig. 7B), *CXCL8* (Fig. 7C) and *DEFBA4* (Fig. 7D) mRNA induction is expressed as fold increase from untreated control (dashed line) after normalization on two reference genes, *ATP5b* and *RPL13a*. Differences were considered statistically significant at * $p < 0.05$ and ** $p < 0.01$ using One-way ANOVA with Games-Howell post-hoc test.

such diesel emissions may enhance the activation of the integrated stress response and inhibits the expression of endoplasmic reticulum chaperone HSPA5/BiP induced by bacterial exposure. These findings provide further information on the mechanisms underlying the adverse effects of traffic-related air pollution, with specific relevance for COPD patients.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Authors have no actual or potential conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tiv.2018.01.024>.

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