ESTROGENIC AND DIOXIN-LIKE ACTIVITY IN DIESEL EXHAUST

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Introduction

Endocrine disrupting chemicals (EDCs) interfere with the hormone system and thereby they cause adverse health effects in humans and animals[1]. Inhalation of EDCs may represent an important exposure pathway that should be considered as a potential health risk. However, the endocrine disrupting potential of airborne compounds is not thoroughly examined.

Our current project aims to provide insights into the exposure of humans and animals to EDCs in the air. To this end, biological methods are combined with chemical analysis to detect and characterize endocrine disruptors in airborne samples (e.g., diesel exhaust, ambient air samples). Chemically activated luciferase expression (CALUX®) bioassays are implemented to screen for estrogenic activity mediated by the estrogen receptor (ER-CALUX®, estrogen responsive assay) and for dioxin-like activity mediated by the aryl hydrocarbon receptor (DR-CALUX®, dioxin responsive assay). Although the aryl hydrocarbon receptor (AhR) is traditionally not seen as a hormone receptor, AhR-ligands may directly or indirectly modulate multiple endocrine signalling pathways[2].

In this short paper, we present data on the endocrine disrupting potential of diesel exhaust. These data are supported by a detailed description of methods and QA/QC parameters employed.

Materials and Methods

Sampling. Exhaust samples were generated with a diesel engine with direct injection (Liebherr, Type D914T, 6.11 litre; engine rating: 110 kW, 2000 RPM, RPM = rounds per minute). Commercially available diesel fuel was used in all test cycles (specification SN 181190-1:2000, class D). The engine was operated according to the ISO 8178/4 C1 cycle valid for construction site engines. This cycle starts with four load-states at maximum RPM (2000), followed by three load-states at an intermediate RPM (60% of rated RPM) and ends with an idling phase. The total cycle time is 100 minutes. In our project, the cycle was driven two times without interruptions. Thus, the duration of a sampling arrangement was 200 minutes. During the two consecutive driving cycles, mass flow proportional aliquots of the exhaust gas were collected on the different load stages (yielding a total volume of 4.4-6.7 m^3).

Complete exhaust samples including particles as well as non-volatile and semi-volatile compounds were collected with an all-glass device that consisted of a heated sampling probe, a heated filter stage, an energy cooler, a condensate separator and a two stage adsorber unit (XAD-2). The glass apparatus employed was cleaned carefully and additionally heated in a high temperature oven to prevent against possible chemical contamination. Prior to sampling, ^{13}C-labeled standards for chemical analysis, which had no activity in the ER- and DR-CALUX® bioassays, were spiked to the condensate separator.

In order to receive a blank control, an additional sampling device was prepared for collection but was not exposed to exhaust gas. As average sample volume, 5.8 m^3 was used to calculate hypothetical concentrations in the blank.

Extractions. All solvents were pro analysis quality or better and were purchased from Merck (Darmstadt, Germany) or Biosolve (Valkenswaard, Netherlands). After sampling, all glass parts of a sampling device were thoroughly rinsed with acetone, toluene and dichloromethane. The rinsing liquids were concentrated, united with the filter and adsorbens of the sampling device and extracted in toluene for 24 hours using a Soxhlet apparatus. Extracts were filtered, dried with Na_2SO_4 and concentrated. Five percent (m/m) of the total extract of each sample and the blank control were separated for testing in the bioassays. The other 95% (m/m) were used for chemical analysis of several toxicologically relevant pollutants, of which sample preparation and analysis data are not shown here.

The 5% aliquots were adjusted with toluene to a volume of 20 ml. A portion of each aliquot was transferred to a glass vial (300 µl or 2 ml). Toluene was evaporated at 50°C under a gentle flow of N_2-gas. Just at the point of dryness, 10 µl of dimethylsulfoxide (DMSO; extra pure, ≥ 99%, Merck, Darmstadt, Germany) was added to samples intended for the ER-CALUX® assay and 1200 µl to samples intended for the DR-CALUX® assay. Samples in DMSO were stored in the dark at 4°C, if they could not be tested immediately in the bioassays.

ER-CALUX® assay. The estrogenic activity (i.e., estrogen receptor ligands/agonists) in diesel exhaust samples was determined in the Estrogen Responsive-Chemically Activated Luciferase Expression assay (ER-CALUX®) received from BioDetection Systems (BDS, Amsterdam, Netherlands). The assay is based on a human adenocarcinoma T47D cell line that is stably transfected with a plasmid containing firefly (*Photinus pyralis*) luciferase gene under control of estrogen responsive elements (pEREtata-Luc)[3]. The cultivation of stably transfected T47D.Luc cells and the performance of the ER-CALUX® assay were carried out following the protocol of BDS with cell culture products from Gibco (Paisley, Scotland) unless stated otherwise. Briefly, T47D.Luc cells were cultured in D-MEM/F-12 growth medium with phenol red supplemented with $NaHCO_3$ (1.25 g/L), 1% (v/v) non-essential amino acids and 7.5% (v/v) fetal bovine serum (FBS). Cells were incubated at 37°C in a 5% (v/v) CO_2 atmosphere with saturated humidity. To perform the ER-CALUX® assay, T47D.Luc cells were plated in 96-well microtiter plates (Nunc, Rochester, USA) at a density of 10,000 cells per well and were maintained in 0.1 ml D-MEM/F-12 phenol red free medium. The medium was supplemented with $NaHCO_3$ (1.26 g/L), 1% (v/v) non-essential amino acids and 5% (v/v) DCC (dextran-coated charcoal)-stripped FBS of Australian origin. After 24 h of incubation (37°C, 5% CO_2), the medium was refreshed and cells were incubated for another 24 h. The medium was removed and cells were exposed to 0.1 ml medium containing diesel exhaust extracts in DMSO, which resulted in 0.1% (v/v) DMSO per well. All samples were tested in triplicate per 96-well-microtiter plate. After 24 h of exposure, medium was removed and 50 µl of lysis reagent (Promega, Madison, USA) was added to each well. If subsequent measurement was not possible, microtiter plates were stored at -20°C for a maximum of two weeks. Prior to measurement, plates were shaken at room temperature for 10 min or for 20 to 30 min, if they had to be thawed. The amount of estrogen receptor agonists in the tested samples was indirectly measured in a luminometer (MLX Microplate Luminometer, Dynex, Chantilly, USA) via the chemiluminescent transformation of luciferin by luciferase. To each well, 100 µl of D-luciferin (Synchem, Kassel, Germany) containing flash-mix (20 mM tricine, 1.07 mM $[MgCO_3]_4Mg[OH_2]\cdot 5H_2O$, 2.67 mM $MgSO_4\cdot 6H_2O$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM co-enzyme A, 470 µM D-luciferin, 530 µM ATP; pH = 7.8) were injected to start the light emission. Subsequently, 100 µl of 0.2 M NaOH solution were added to stop the chemiluminescent reaction. The measurements were reported in relative light units (RLU) as the total integral of light intensity during an integration window of 10 s. After correction for background signal (DMSO solvent control), the estrogenic activity of the samples was recalculated into 17β-estradiol (E2) equivalents by interpolating the ER-CALUX® response in a simultaneously tested E2 calibration curve (standard dilution series: BDS; sigmoidal curve $y = a_0/[1 + (x/a_1)\text{^}a_2]$, y = measured RLUs, x = E2 concentration in well, a_0 = maximum RLU response, $a_1 = EC_{50}$, a_2 = slope factor; user-defined curve fit, $r^2 \geq 0.98$). Finally, the activities were expressed as E2 mass equivalents per m^3 diesel exhaust. Quantification into E2 mass equivalents was only done for ER-CALUX® responses that were between the limit of quantification (LOQ, 1.5 pM in well) and the 50% effect concentration (EC_{50}) of the calibration curve. To produce quantifiable ER-CALUX® responses, preliminary range-finding experiments were performed. In so doing, adequate dilution factors could be determined for each diesel exhaust sample.

DR-CALUX® assay. The dioxin-like activity (i.e., amount of Ah-receptor ligands/agonists) in diesel exhaust samples was determined using the Dioxin Responsive-Chemically Activated Luciferase Expression assay (DR-CALUX®) licensed by BioDetection Systems (BDS, Amsterdam, Netherlands). The DR-CALUX® assay uses a rat hepatoma H4IIE cell line stably transfected with firefly luciferase gene under the control of dioxin responsive elements (pGudLuc1.1)[4]. If not stated otherwise, all liquids for cell culture and assay performance were purchased from Gibco (Paisley, Scotland). According to the BDS protocol, stably transfected H4IIE.Luc cells were cultivated in MEM-α medium supplemented with 10% (v/v) FBS at 37°C in a 5% (v/v) CO_2 atmosphere with saturated humidity. The DR-CALUX® assay was performed using H4IIE.Luc cells in 96-well microtiter plates (Nunc, Rochester, USA) as described in the BDS protocol. After 24 h of incubation (0.1 ml MEM-α with 10% FBS per well; 37°C, 5% CO_2), medium (0.1 ml) containing diesel exhaust extracts in DMSO was added to each well. Cells were exposed in triplicate for 24 h at a solvent concentration of 0.8% (v/v) DMSO. Then, medium was removed, cells were washed with 100 µl phosphate-buffered salt solution and harvested using 50 µl

of lysis reagent (Promega, Madison, USA). If immediate analysis was not possible, plates were stored at -20°C for a maximum of two weeks. The amount of aryl hydrocarbon receptor ligands in the tested samples was indirectly measured in a luminometer as described for the ER-CALUX® assay. For the calculation of induced responses, a concentration series of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was included on each microtiter plate. The dioxin-like activity of the samples was recalculated into TCDD mass equivalents per m^3 diesel exhaust in the same way as described for E2 mass equivalent calculations (ER-CALUX® assay). The quantifiable range was set between the LOQ (1 pM in well) and the EC_{50} of the calibration curve. Quantifiable responses were achieved through range finding experiments.

QA/QC. For background correction, a DMSO solvent control for the samples and a DMSO solvent control for the standard dilution series were included on each 96-well microtiter plate. An internal reference material (plate IRM) and the 3 pM calibration point were used to control the assay performance (i.e., to ensure reliable results). The calculated E2 and TCDD mass equivalents of the plate IRM had to be equal to a previously determined average plate IRM value ± 2 times the standard deviation. The plate IRM consisted of a 17α-ethinylestradiol (Fluka, Buchs, Switzerland) solution in DMSO (ER-CALUX®) and a PCB congener mixture (AccuStandard, New Haven, USA) transferred into DMSO (DR-CALUX®), respectively.

Several quality criteria had to be fulfilled on each microtiter plate to allow interpolation of samples in the calibration curve. The induction factor (IF) for the calibration curve ($IF = (a_0 + RLU_{DMSO})/RLU_{DMSO}$) had to be equal to 6 or higher. Quantification was only allowed in the concentration range between the LOQ and EC_{50} of the calibration curve. The correlation coefficient (r^2) for the calibration curve had to be equal or higher than 0.98.

Results and Discussion

Endocrine disruptors in diesel exhaust. Diesel engines emit inhalable fine particulate matter of chemical composition still not fully characterized[5] and countless known and unknown air pollutants. Some of those compounds are potential endocrine disrupting chemicals (EDCs). In the present study, the endocrine activity of diesel exhaust was investigated using the ER- and DR-CALUX® bioassays. We measured an average of 1.5 ± 0.2 ng 17β-estradiol equivalents (n=8) and 60 ± 14 ng 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (n=9) per m^3 diesel exhaust. As samples were not cleaned in a column with sulfuric acid-silica, unstable compounds (e.g., PAHs) may contribute considerably to the DR-CALUX® response. Figure 1 shows that the tested diesel exhaust sample exhibits an order of magnitude higher estrogenic activity and two orders of magnitude higher dioxin-like activity than the blank control.

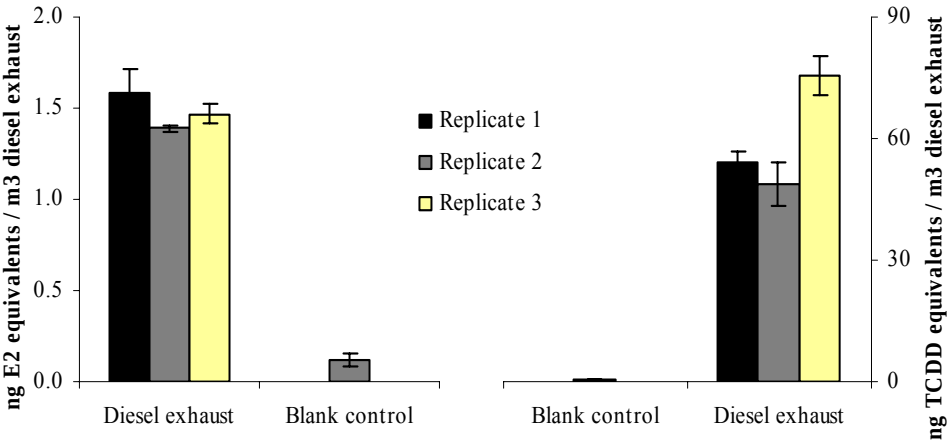

Figure 1. A diesel exhaust sample was investigated in the ER- and DR-CALUX® bioassay. The endocrine activity is reported as ng 17β-estradiol (E2) equivalents per m^3 diesel exhaust and as ng 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents per m^3 diesel exhaust, respectively. The diesel exhaust sample was measured in triplicate (error bars) and on three separate days (replicate 1-3).

Our study shows that diesel exhaust exhibits both, estrogenic and dioxin-like activity in the CALUX® assays. Thus, diesel engines are a source of airborne estrogen receptor- and aryl hydrocarbon receptor-ligands that may interfere with the hormone system of humans and animals. Evidence for EDCs in diesel exhaust is reported in other studies using different in vitro test systems (e.g., Meek[6]) or in vivo assays (e.g., Watanabe and Kurita[7]).

Potential toxicological relevance. The activity of endocrine disruptors can result from several distinct mechanisms[1]. Binding to an intracellular receptor is one of them. Therefore, CALUX® assays are a useful screening method to detect chemicals that bind to a receptor like an endogenous hormone (e.g., E2) or a well studied endocrine disruptor (e.g., TCDD). Unfortunately, results of in vitro bioassays cannot provide a clear one-to-one link to endocrine effects in humans and animals. They analyse effects at the level of cells and macromolecules and therefore they cannot reflect in vivo effects. Especially, cell tests do not account for intake and metabolism in an intact organism.

Outlook. Diesel-powered vehicles are known to contribute appreciable amounts of inhalable fine particulate matter to the atmosphere of urban areas[5]. Therefore, particulate traps were developed to efficiently reduce the emission of particulate matter[8]. Studies have shown that some fuel additive-regenerated particulate traps can lead to the formation of toxic secondary emissions such as dioxins or Nitro-PAHs[8]. To assess the effect of particulate traps on the emission of potential EDCs, diesel exhaust samples collected with and without exhaust gas after-treatment will be tested in the ER- and DR-CALUX® assay. Data presented in this short paper are from a diesel exhaust sample collected without particulate trap.

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