Ultrafine carbon particles down-regulate CYP1B1 expression in human monocytes

Christiane Eder1, Marion Frankenberger1, Franz Stanzel2,5, Albrecht Seidel3, Karl-Werner Schramm4, Loems Ziegler-Heitbrock1 and Thomas PJ Hofer*1

Address: 1Helmholtz Zentrum Muenchen, German Research Center for Environmental Health, Clinical Cooperation Group Inflammatory Lung Diseases, Institute of Lung Biology and Disease and Asklepios Fachkliniken Muenchen-Gauting, Robert-Koch-Allee 29, 82131 Gauting, Germany, 2Asklepios Fachkliniken Muenchen-Gauting, Robert-Koch-Allee 2, 82131 Gauting, Germany, 3Biochemisches Institut für Umweltcarcinogene, Lurup 4, 22927 Grosshansdorf, Germany, 4Helmholtz Zentrum Muenchen, German Research Center for Environmental Health, Institute for Ecological Chemistry, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany and 5Current address: Lungenklinik Hemer, Theo-Feuncius-Strasse 1, 58675 Hemer, Germany

Email: Christiane Eder - christiane.eder@mll-online.de; Marion Frankenberger - frankenberger@helmholtz-muenchen.de; Franz Stanzel - franz.stanzel@lhemmer.de; Albrecht Seidel - albrecht.seidel@biu-grimmer.de; Karl-Werner Schramm - schramm@helmholtz-muenchen.de; Loems Ziegler-Heitbrock - ziegler-heitbrock@helmholtz-muenchen.de; Thomas PJ Hofer* - hofer@helmholtz-muenchen.de

* Corresponding author

Abstract

Background: Cytochrome P450 monooxygenases play an important role in the defence against inhaled toxic compounds and in metabolizing a wide range of xenobiotics and environmental contaminants. In ambient aerosol the ultrafine particle fraction which penetrates deeply into the lungs is considered to be a major factor for adverse health effects. The cells mainly affected by inhaled particles are lung epithelial cells and cells of the monocyte/macrophage lineage.

Results: In this study we have analyzed the effect of a mixture of fine TiO2 and ultrafine carbon black Printex 90 particles (P90) on the expression of cytochrome P450 1B1 (CYP1B1) in human monocytes, macrophages, bronchial epithelial cells and epithelial cell lines. CYP1B1 expression is strongly down-regulated by P90 in monocytes with a maximum after P90 treatment for 3 h while fine and ultrafine TiO2 had no effect. CYP1B1 was down-regulated up to 130-fold and in addition CYP1A1 mRNA was decreased 13-fold. In vitro generated monocyte-derived macrophages (MDM), epithelial cell lines, and primary bronchial epithelial cells also showed reduced CYP1B1 mRNA levels. Benzo[a]pyrene (BaP) is inducing CYB1B1 but ultrafine P90 can still down-regulate gene expression at 0.1 μM of BaP. The P90-induced reduction of CYP1B1 was also demonstrated at the protein level using Western blot analysis.

Conclusion: These data suggest that the P90-induced reduction of CYP gene expression may interfere with the activation and/or detoxification capabilities of inhaled toxic compounds.

Background

Several epidemiologic studies attribute increased morbidity and mortality to exposure to environmental particles [1-3]. These adverse health effects due to the inhalation of particulate matter are a topic of ongoing scientific and public concern. Particulate matter (PM) is a complex mixture of many different components, which can be characterized by origin (anthropogenic or geogenic), by...
Physicochemical properties (such as solubility) or by particle size. Particles with a mean aerodynamic size between 10 and 2.5 μm (PM<sub>10</sub>) are classified as coarse particles, fine particles have a size between 2.5 and 0.1 μm and particles with a diameter less than 0.1 μm are termed ultrafine. Not only the particle size but other particle-associated parameters like particle number, surface area or reactive compounds adsorbed to the surface may be involved in the observed health effects [4]. Because of their small size, ultrafine particles contribute only modestly to total mass, but they are the predominant fraction by number in PM. Most urban particles result from combustion processes, therefore the major fraction contains ultrafine carbonaceous particles [5]. After deposition in the lung larger particles are phagocytized by alveolar and airway macrophages [6,7], but the fine and ultrafine carbon particles remain in the lung for a longer period of time [5]. Ultrafine particles are phagocytized to a minor extend but they can still enter macrophages and epithelial cells and even penetrate into the circulation. Thus ultrafine particles not only trigger local inflammatory reactions in the lung but also cause systemic extrapulmonary effects [8]. Ultrafine particles also have the capacity to inhibit phagocytosis by alveolar macrophages [9]. Macrophages and their monocyte progenitors are major elements of the inflammatory response. In addition to performing phagocytosis they can release inflammatory mediators such as cytokines and chemokines and they are crucially involved in destruction of microbes and particles using various enzymatic systems [10]. Cytochromes like CYP<sub>1B1</sub> are also expressed by macrophages and these enzymes are part of the "digestive" and detoxifying machinery of these cells [8].

The xenobiotic metabolism can be divided into two phases: modification (phase I) and conjugation (phase II). An important group of phase I enzymes consists of the cytochrome P450 oxidases (CYP) which belong to the monooxygenases. In humans 57 CYPs are known and about 25% of them are considered to be involved primarily in the xenobiotic metabolism [11]. Superfamily members are classified according to the similarity of their primary structure. The expression of the CYP<sub>1</sub> subfamily can be induced by polycyclic aromatic hydrocarbons (PAH), which are ubiquitously occurring environmental carcinogens [12] and are particularly known to be present in cigarette smoke [13]. The induction of CYP<sub>1</sub> genes is regulated by a heterodimer of the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator (Amt) [14]. Two cytochromes, CYP<sub>1A1</sub> and CYP<sub>1B1</sub>, are mainly involved in the formation of ultimate carcinogenic diol-epoxides of PAH such as benzo[a]pyrene (BaP) [15]. The expression of these enzymes is largely extrahepatic and both enzymes are present in many tumor tissues [16,17]. CYP<sub>1B1</sub> has been identified as a major P450 enzyme in normal human blood monocytes [18] and CYP<sub>1B1</sub> is also present in human lung and lung-derived cell lines [19].

Monocytes, macrophages and epithelial cells are affected by particles. CYP<sub>1B1</sub> is involved in both, detoxification as well as metabolic activation of xenobiotics [12]. Thus it is important to address the question of whether and in what way particles affect CYP<sub>1B1</sub>. This study is aimed on the effects of carbon particles on the expression of CYP<sub>1B1</sub> in monocytes/macrophages and bronchial epithelial cells and we demonstrate a pronounced down-regulation of mRNA expression and protein level of this important extrahepatic enzyme.

**Results**

**Effect of particle exposure on CYP1B1 mRNA expression**

In earlier experiments using gene expression arrays, we noted a decreased expression of CYP<sub>1B1</sub> mRNA in monocyte-derived macrophages (MDM) of patients with COPD [http://www.ncbi.nlm.nih.gov/geo/ accession number GSE8608]. Since exposure of particles plays a major role in the etiology of this disease, we studied the effect of particles on cells of the monocyte/macrophage lineage. To cover a wider range of particle materials (chemistry) and their physical properties (size, surface structure) and because of economical reasons (cost-effectiveness) we initially used a mixture of both particles, ultrafine P90 (mean size 90 nm) and fine TiO<sub>2</sub> (mean size 200 nm). We analyzed the effect of this mixture on CD14<sup>+</sup> monocytes after 3h of exposure.

Expression levels were calculated relative to corresponding mRNA level for α-enolase in the same sample, such that negative values indicate a transcript prevalence that is less than that of α-enolase in the same cell population. As shown in Fig. 1A, incubation of CD14<sup>+</sup> monocytes with this mixture of ultrafine P90 and fine TiO<sub>2</sub> resulted in a pronounced decrease of CYP<sub>1B1</sub> mRNA transcripts (-4,308 ± 2,231) reflecting a 95-fold reduction compared to untreated cells (-45 ± 37). LPS showed a minor effect with respect to CYP<sub>1B1</sub> expression (-68 ± 41).

We then addressed the question which of the two types of particles in the mixture caused the down-regulation of CYP<sub>1B1</sub> mRNA in CD14<sup>+</sup> monocytes. As shown in Fig. 1A the decrease of CYP<sub>1B1</sub> transcripts can be attributed to ultrafine P90 alone which reduced expression 136-fold to a level of -6,109 ± 1,759, while the fine TiO<sub>2</sub> showed no effect (-50 ± 21) compared to untreated cells (-45 ± 37).

Since the down-regulation of CYP<sub>1B1</sub> is only found in P90 we next asked whether this was due to the ultrafine nature of the particle. We therefore tested ultrafine TiO<sub>2</sub> but found no activity with an expression level of -34 ± 20 in untreated and of -27 ± 18 in treated PBMC (n = 3; data not shown). Hence low size is not sufficient a feature to
monocytes with ultrafine P90 for 3 h led to a pronounced 13-fold down-regulation of CYP1A1 mRNA (-12,204 ± 5,622). No effect of LPS (-1,508 ± 781) or fine TiO2 (-1,013 ± 746) was detected when compared to untreated cells (-940 ± 823) (Fig. 1B). These data show that CYP1A1 is also affected by ultrafine P90 albeit to a lesser extent compared to CYP1B1.

**Exclusion of LPS-contamination of particles**

A frequent problem in cell biology is LPS-contamination of materials including particles such as those used in our experiments. While in our system LPS alone had a minor effect on its own, we still needed to exclude a contribution of this compound when combined with particles. For this we used Polymyxin B, a compound which is known to inhibit pro-inflammatory signals induced by LPS [20]. Polymyxin B on its own did not alter mRNA expression of CYP1B1 (-10 ± 3) compared to untreated cells (-12 ± 4). It did suppress however the moderate LPS-induced decrease of CYP1B1 mRNA from -24 ± 10 to -13 ± 9 (p < 0.05). On the other hand the P90-induced down-regulation of expression of CYP1B1 mRNA (-1,292 ± 1,031) was not altered by Polymyxin B (-945 ± 935) (not significant, Fig. 2). These findings support the assumption that particles are not contaminated by LPS.

**Dose response and time course of P90-induced CYP1B1 mRNA repression**

To determine optimum dose of ultrafine carbon particles for cytochrome monooxygenase 1B1 mRNA repression we incubated CD14++ monocytes with or without different doses of P90 for 3 h. Subsequently CYP1B1 mRNA levels were detected (Fig. 3A). The effects were significant even at low doses, starting with 0.32 μg/ml (-18 ± 8), followed by 3.2 μg/ml (-69 ± 41). A more pronounced decrease of CYP1B1 transcripts was seen at 32 μg P90/ml (-1,152 ± 882, 96-fold reduction), at 320 μg P90/ml (2192 ± 1173, 183-fold reduction) and at 1,000 μg/ml (-2,814 ± 1,754, 235-fold reduction). To exclude a toxic effect on cells incubated with the high particle concentrations, a trypan blue viability test was performed. It showed no decrease of cell viability at all particle concentrations. Levels of α-enolase mRNA also gave no evidence of loss of viability of these cells (data not shown). For all further experiments we used a particle concentration of 32 μg/ml dose.

Next we analyzed the time course of CYP1B1 mRNA repression (Fig. 3B). CD14++ monocytes were incubated with P90 at 32 μg/ml from 0.5 h to 20 h. Repression of CYP1B1 was detectable after 0.5 h incubation (-27 ± 2), was more pronounced after 1 h (-89 ± 18) and reached a plateau after 3 and 6 h (-1,856 ± 605, 116-fold reduction, and -1,766 ± 584, 110-fold reduction, respectively). After 20 h CYP1B1 mRNA levels recovered but were still 46-fold reduced (-740 ± 275) compared to untreated cells. Based on these results, a dose of 32 μg/ml and 3 h incubation were sufficient to measure the repression of CYP1B1 mRNA by ultrafine P90.

**Figure 1**

A) Effect of LPS, ultrafine P90, and fine TiO2 on CYP1B1 mRNA levels in CD14++ monocytes. Cells were purified from PBMC of healthy donors by MACS separation. CD14++ cells remained untreated (none) or were stimulated with LPS (10 ng/ml), with particle-mix of ultrafine P90 and fine TiO2 (each with 32 μg/ml) or with each particle separately for 3 h. Cells were lysed and mRNA levels were determined by RT-PCR. Data were normalized to levels of the house keeping gene α-enolase. (n = 3 incubations from different donors, mean ± S.D.; * p < 0.05 compared to untreated controls). B) Down-regulation of CYP1A1 mRNA in monocytes. CD14++ cells remained untreated (none) or were stimulated with LPS (10 ng/ml), with particle-mix of ultrafine P90 and fine TiO2 (each with 32 μg/ml) or with each particle separately for 3 h. Cells were lysed and mRNA levels were determined by RT-PCR. (n = 3 incubations from different donors, mean ± S.D.; * p < 0.05 compared to untreated controls).

explain the effect on CYP1B1. Other properties found in P90 and not in TiO2 appear to be responsible.

CYP1A1 is a cytochrome monooxygenase closely related to CYP1B1, so we asked whether expression of this gene is also influenced by particles. Incubation of CD14++
on this analysis we used the 3 h time point for all further experiments.

**Effect of particles on CYP1B1 and CYP1A1 mRNA expression in MDM from healthy donors and COPD patients**

MDMs are more mature than monocytes and might show an effect of ultrafine P90 on CYP1B1 expression different from what is seen in monocytes. We therefore matured freshly isolated CD14++ monocytes with M-CSF at 100 ng/ml for 5 days and subsequently treated the cells with 32 μg P90/ml for 3 h (Fig. 4A). We used cells from healthy donors and COPD patients to address the question whether these patients have an altered capacity to deal with exogenous particulates.

In untreated MDM of healthy donors a basal mRNA expression of CYP1B1 was at -40 ± 40. After LPS stimulation CYP1B1 transcript was reduced by factor 2.6 (-105 ± 114, Fig. 4A). Stimulation with the particle-mix of ultrafine P90 and fine TiO2 led to a 2.7-fold reduction of CYP1B1 mRNA (-109 ± 87, Fig. 4A). Untreated MDM from COPD patients showed a higher level of CYP1B1 mRNA (-27 ± 11) and a 2-fold decrease in CYP1B1 transcript after incubation with particles (-56 ± 21, Fig. 4A).

Looking at CYP1A1 expression in the same MDM we noted a much lower level or constitutive expression compared to CYP1B1. In healthy donors CYP1A1 mRNA expression was -19,350 ± 17,811, after LPS and particle treatment IA1 transcript levels were -84,195 ± 49,677 (4-fold) and -55,720 ± 96,911 (3-fold) respectively. In MDM of COPD patients IA1 mRNA levels were -66,840 ± 59,479 in untreated cells and -83,509 ± 66,575 in particle-treated cells (not significant; data not shown).
A) MDM were generated from CD14++ monocytes purified from PBMC by MACS separation followed by 5-day incubation with M-CSF (100 ng/ml). Cells remained untreated (none) or were stimulated with LPS (10 ng/ml) or with particle-mix of ultrafine P90 and fine TiO2 (each with 32 μg/ml) for 3 h (n = 5 patients with COPD, n = 8 healthy controls, mean ± S.D.; * p < 0.05 compared to untreated controls).

B) Effect of particles on CYP1B1 expression in sputum macrophages of healthy non-smokers, healthy smokers and patients with COPD. Sputum macrophages were purified using RosetteSep to deplete unwanted leukocytes. Cells remained untreated (none) or were stimulated with LPS (10 ng/ml) or with particle-mix of ultrafine P90 and fine TiO2 (each with 32 μg/ml) for 3 h (n = 5 non-smokers, n = 4 smokers, n = 7 patients with COPD, mean ± S.D.; * p < 0.05).

C) Cells remained untreated (none) or were stimulated with LPS (10 ng/ml) with particle mix of ultrafine P90 and fine TiO2 (each with 32 μg/ml) or with each particle separately for 22 h (each with 32 μg/ml) (A549 n = 3, Calu-3 n = 4 experiments from different cell passages, mean ± S.D.; * p < 0.05 compared to untreated control).

D) Effect of ultrafine P90 on CYP1B1 mRNA expression in primary bronchial epithelial cells. Cells were obtained by bronchial brush biopsy. Cells remained untreated (none) or were stimulated with ultrafine P90 (32 μg/ml) for 3 h (n = 7 incubations from different donors, mean ± S.D.; * p < 0.05 compared to untreated control).
Taken together MDMs when compared to blood monocytes do show a decrease of cytochrome monooxygenases but the effect appears to be less pronounced with respect to CYP1B1 and CYP1A1.

**Effect of particles on CYP1B1 mRNA expression in sputum macrophages from non-smokers, smokers, and COPD patients**

Macrophages in the airways are exposed to inhaled particles and this may impact on CYP expression. We therefore isolated macrophages from induced sputum of healthy non-smokers, healthy smokers and COPD patients (6 ex-smokers and one current smoker). In healthy non-smokers particles reduced CYP1B1 expression in sputum macrophages 2.8-fold (-530 ± 547) compared to untreated cells (-191 ± 198) (Fig. 4B). In COPD expression of CYP1B1 was at -90 ± 97. Treatment of COPD sputum macrophages for 3 h with ufP90/TiO2 led to a 2.7-fold decrease of CYP1B1 transcripts (-247 ± 346) compared to 3 h untreated cells. The single currently smoking patient showed the highest mRNA expression of CYP1B1 (-22) with no decrease of transcript after ultrafine P90/fine TiO2 stimulation (-29).

When looking at healthy smokers we found a very high expression level for CYP1B1 (-18 ± 15). Incubation with the particle mixture for 3 h had no effect on CYP1B1 transcripts of smokers (-16 ± 13) compared to untreated cells (Fig. 4B). These data show that in smokers sputum macrophages are refractory to the action of particles.

**Effect of particles on CYP1B1 mRNA expression in human epithelial cells**

Inhaled particles also deposit on alveolar epithelial cells. We thus investigated the effect of ultrafine P90 and fine TiO2 on CYP1B1 mRNA expression in the human alveolar epithelial cell line A549 and a bronchial epithelial cell line Calu-3. In these experiments we used an incubation time of 22 h as a preliminary time point, which afterwards was optimized depending on cell type (primary cells or cell line) and read-out (transcript or protein analysis).

Compared to untreated A549 cells (-158 ± 73) CYP1B1 mRNA levels were decreased after 22 h incubation with P90 by factor 4 (-707 ± 347, p < 0.05) (Fig. 4C). LPS (-206 ± 81) treatment shows no effect, whereas a slight effect was observed with fine TiO2 (-287 ± 134) treatment. In cells incubated with both ultrafine P90 and fine TiO2, CYP1B1 transcript levels were decreased to -948 ± 507.

In the bronchial epithelial cell line Calu-3 the expression of CYP1B1 in untreated cells was 301 ± 140 (Fig 4C). Ultrafine P90- (-2,820 ± 1,546) and ultrafine P90/fine TiO2-treated cells (-3,106 ± 1,988) showed a strong 10-

fold decrease of CYP1B1 mRNA. LPS (-360 ± 147) and fine TiO2 (-297 ± 144) showed no effect.

To confirm the results of epithelial cell lines we investigated the effects of P90 on CYP1B1 mRNA expression in primary bronchial epithelial cells. Cells were obtained by bronchial brush and treated with and without 32 μg ultrafine P90/ml for 3 h. As shown in Fig. 4C CYP1B1 transcript levels were reduced 4-fold in ultrafine P90-treated cells (-1,163 ± 1,161) compared to untreated cells (-267 ± 373). This included two cases with a very strong response (35- and 14-fold). Hence primary epithelial cells can be very sensitive to the action of ultrafine carbon particles.

**Effect of benzo[a]pyrene on CYP1B1 mRNA expression in human peripheral blood mononuclear cells (PBMC)**

Cytochrome P450 enzymes are known to be involved in the metabolism of polycyclic aromatic hydrocarbons (PAH). Benzo[a]pyrene (BaP), a carcinogenic representative of this class of compounds, strongly up-regulated CYP1B1 mRNA levels in PBMC. Time course experiments showed an increase in transcripts from -88 ± 20 in untreated cells to -6 ± 2 in cells after 3 h, and -4 ± 2 after 6 h incubation with BaP (data not shown).

To investigate whether BaP is capable of overcoming the repression of CYP1B1 induced by P90, PBMC were either pre-incubated with ultrafine P90 particles (32 μg/ml) or remained untreated. After 30 min of incubation, BaP-con-
CYP1B1 mRNA in untreated cells (-29 ± 18) was decreased by ultrafine P90 treatment (-1,178 ± 1,011), on the other hand BaP induced this gene (0.1 μM: -11 ± 4.2; 1 μM: -8.5 ± 3.7; 10 μM: -8.1 ± 3.8). While at high BaP concentrations (1 and 10 μM), ultrafine P90 could not reduce CYP1B1 mRNA, it was able to suppress the transcripts by factor 7 at 0.1 μM BaP (p < 0.05).

**Effect of P90 on CYP1B1 protein levels in Calu-3**

To investigate CYP1B1 protein levels we performed Western blot analysis with isolated microsomal protein of Calu-3 cells. In the blot shown in Fig. 6, the densitometric reading for CYP1B1 protein in Calu-3 decreased from 3,036,276 AU (untreated cells, 32 h) to 373,799 AU after ultrafine P90-treatment for 32 h. In average of 3 experiments CYP1B1 protein level in untreated cells was 2,721,541 ± 379,059 AU and P90 treatment reduced this to 342,109 ± 46,303 AU (Fig. 6).

These data show that P90 will lead to a pronounced reduction of CYP not only at the mRNA but also at the protein level.

**Discussion**

The data of this study show that P90 causes a strong down-regulating effect on the CYP1B1 expression. Cytochrome P450 monooxygenases are involved in detoxification and toxification of xenobiotic substances. Toxification is caused by metabolic transformation of non or less toxic precursors into reactive intermediates. Cells may thus be influenced by two mechanisms/modes of action, they may either be protected or they may become more susceptible to other inhaled substances by exposure to ultrafine P90.

The strongest down-regulation of CYP1B1 expression after stimulation with particle mix of ultrafine P90 and fine TiO₂ was observed in monocytes (60-fold, Fig. 1A). It is unclear at this point why CYP1B1 is so much more sensitive to P90 effects than CYP1A1 (Fig. 1B). Whether this is determined at the promoter level needs to be addressed in the future. As P90 was identified as the active component in the particle mix (Fig. 1A) we addressed two questions.

The first question was, whether ultrafine TiO₂ particles were capable to show such strong down-regulation of CYP1B1 mRNA expression as seen with ultrafine P90. To study if the strong P90 effect is triggered by its ultrafine nature (12 nm in diameter, specific surface area of 300 m²/g), we incubated cells with ultrafine TiO₂ with a diameter of 20 nm and a specific surface area of 48 m²/g [5]. Neither fine nor ultrafine TiO₂ treatment did alter CYP1B1 mRNA expression. Beck-Speier et al. have shown a highly significant correlation between the PGE_2/TXB_2 formation and the specific particle surface area but not the mass concentration [21]. The smaller surface area of ultrafine TiO₂ could be an explanation for the weaker effect of ultrafine TiO₂ (48 m²/g) compared to ultrafine P90 (300 m²/g). Also the chemical composition or surface structure of the particles may contribute to their reactivity towards various
cell types. Dick et al. [22] found for ultrafine TiO$_2$ and ultrafine P90 purchased from the same manufacturer and with the same composition and surface area than those used herein, different effects in mice when instilled into the lungs. Ultrafine P90 exhibited a higher effect in influx of neutrophile granulocytes into the lungs, higher membrane damage (causes release of G-glutamyl transferase), and higher levels of macrophage inflammatory protein 2 (MIP-2) in the lavage fluid 48 h after instillation than for ultrafine TiO$_2$.

Secondly we addressed the question, whether the observed effect was caused by an LPS contamination of P90. LPS contamination can be excluded by neutralizing LPS with Polymyxin B. Our experiments with Polymyxin B showed clearly that the down-regulation of CYP1B1 is caused by ultrafine P90 and not by a potential endotoxin contamination of the carbon black particles (Fig. 2). The LPS-mediated reducing effect on CYP1B1 expression could be abolished by Polymyxin B, whereas the P90 effect was not altered by Polymyxin B treatment.

With increasing concentrations of ultrafine P90 the decrease of CYP1B1 expression becomes stronger. Each of the observed effects was significant. Therefore the P90 concentration of 32 $\mu$g/ml was used for all further experiments. The concentration of 32 $\mu$g/ml is in accordance with environmentally relevant particle concentration. Higher concentrations do not reflect realistic physiological conditions [23].

We confirmed the reduced CYP1B1 mRNA-expression in additional cell types. In MDM (Fig. 4A) we observed a 2.7-fold decrease of CYP1B1 after particle treatment. In sputum macrophages of healthy non smokers (Fig. 4B) we showed a 4-fold and of COPD patients a 3-fold reduction of CYP1B1. One of the COPD patients currently smoked and showed a high level of CYP1B1 mRNA in untreated sputum macrophages that was not affected after ultrafine P90 treatment. Additionally in healthy smokers no effect of particle treatment on CYP1B1 transcript level was detected. This may be due to the high concentration of organic compounds, e.g. polycyclic aromatic hydrocarbons (PAH), in cigarette smoke which in turn induced CYP1B1 mRNA expression in a competitive manner. A competitive behavior between induction of CYP1B1 mRNA by benzo[a]pyrene (BaP), a carcinogenic constituent of tobacco smoke [24], and decrease of CYP1B1 transcript by ultrafine P90 was clearly shown herein (Fig. 5). Also these findings may suggest that monocytes become less sensitive to ultrafine P90 treatment during matura-

Epithelial cells are also affected by particle exposure. In epithelial cell lines (Fig. 4C) we observed significant down-regulation of CYP1B1 after ultrafine P90 stimulation. Primary epithelial cells obtained by bronchial brush (Fig. 4D) showed in average a 4-fold reduction of CYP1B1 mRNA with strong reductions (35- and 14-fold) in two samples. To exclude a leukocyte contamination we analyzed the cells by flow cytometry and microscopic cell differentiation. The stronger effects seen in two of the seven samples may be due to the inter-individual variation or may be due to the different clinical conditions and medications. Taken together there was no apparent clinical feature (diagnosis, medication, smoking status) to explain the higher responses of bronchial epithelial cells in the two cases.

To confirm the CYP1B1 mRNA down-regulation on protein level, we isolated the microsomal fraction, because CYP1B1 is bound to the endoplasmatic membrane. It was not possible to isolate sufficient numbers of primary cells in order to obtain enough protein for Western blotting. Therefore for Western blot we used the bronchial epithelial cell line Calu-3 as a model cell line because they also show a decrease in CYP1B1 transcripts at mRNA level after ultrafine P90 exposure. The strongest reduction of CYP1B1 mRNA was seen after 22 h, therefore we incubated Calu-3 cell with P90 for 32 h to investigate protein expression. Densitometric analysis of the Western blots confirmed a reduction of CYP1B1 protein following P90 treatment (Fig. 6). We assume that also in monocytes and macrophages CYP1B1 protein will be decreased substantially, similar to the mRNA reduction in these cells.

When alveolar macrophages, monocytes, and airway epithelial cells are exposed to particles, they are phagocytized by these cell types [25,26] and can subsequently interfere with gene expression or cell functions. For cells of the monocytic lineage, one possibility is a transport mechanism of particles via the macrophage receptor with collagenous structure (MARCO). In a study of Kanno et al. an association between the uptake of fine and ultrafine particles and MARCO was shown [27]. In our study we blocked the MARCO receptor with antibodies. The reduction of CYP1B1 transcripts after ultrafine P90 treatment was not altered by blocking antibodies compared to isotype controls (data not shown). Accordingly the transport mechanism of ultrafine P90 particles appears not to be based on the MARCO receptor and the mechanism involved is still elusive.

Transcription of CYP1B1 and CYP1A1 genes is regulated by the interaction between PAH and the cytosolic arylhydrocarbon receptor. Dioxin is the most potent agonist [17], but also BaP can activate the receptor complex [28]. Cigarette smoke and diesel soot contain a huge amount of PAH adsorbed to particles [29,30]. Herein we showed a
BaP-mediated induction of \textit{CYP1B1} in a concentration-dependent manner (Fig. 5). Simultaneously treatment with ultrafine P90 attenuates the \textit{CYP1B1} induction. The attenuation may be less pronounced with low concentration of BaP (0.1 μM) because of absorption effects of BaP onto P90 particles, which reduce the amount of bioavailable BaP. Overall these findings indicate a competing explanation for LPS and cytokine-mediated \textit{CYP} reduction may be an interaction with NFκB [38].

The reduction of \textit{CYP1B1} after ultrafine P90 treatment could be a form of protective mechanism concerning oxidative stress. Reactive oxygen species (ROS) generated by \textit{CYP}-catalyzed metabolisms can cause oxidative stress in cells [39]. Reduced \textit{CYP1B1} may prevent further DNA damage being caused by ROS. Furthermore, decreased availability of \textit{CYP1B1} enzyme leads to a lesser activation of PAH to reactive metabolites which otherwise could cause DNA damage and cancer development [12]. On the other hand \textit{CYP1B1} and \textit{CYP1A1} are also important for detoxifying various xenobiotics. A reduced expression of these cytochromes in monocytes/macrophages and lung epithelial cells induced by inhaled carbon particles may lead to increased damage by xenobiotics because of the lack of its detoxifying character. Further investigations will be required to elucidate by which mechanism the particles affect the \textit{CYP1B1} expression.

\textbf{Methods}

\textbf{Cell lines and culture medium}

The epithelial cell lines A549 [40] and Calu-3 [41] were cultured in Dulbecco’s minimal essential medium NUT mix F12 (21331-020, Invitrogen, Karlsruhe, Germany), containing 10% FCS (S0115, Biochrom, Berlin, Germany), L-glutamine 2 mM (15140-114, Invitrogen, Karlsruhe, Germany), and penicillin 200 U/ml/streptomycin 200 μg/ml (15140-114, Invitrogen, Karlsruhe, Germany).

Primary human cells were cultured in RPMI 1640 medium (F1415, Biochrom, Berlin, Germany), supplemented with OPI (oxaloacetate, pyruvate, insulin; O-5003, Sigma, Taufkirchen, Germany), L-glutamine 2 mM (15140-114, Invitrogen, Karlsruhe, Germany), penicillin 200 U/ml/streptomycin 200 μg/ml (15140-114, Invitrogen, Karlsruhe, Germany), and 1× nonessential amino acids (11140-35, Invitrogen, Karlsruhe, Germany). Before addition of 10% FCS (S0115, Biochrom, Berlin, Germany), the supplemented medium was ultrafiltered through a Gambro 2000 column (Gambro, Hechingen, Germany) in order to remove any inadvertent LPS contamination.

\textbf{Donors of primary human cells}

Primary human cells were obtained from healthy human volunteers (non-smoker and smoker), from patients with COPD, or from patients with other lung diseases (Table...
Written informed consent was obtained from each individual. The protocol was approved by the Ethics Committee of the Medical School of the Ludwigs-Maximilians-University (Munich, Germany).

**Isolation of peripheral blood mononuclear cells (PBMC), enrichment of CD14++ monocytes, and generation of monocyte-derived macrophages (MDM)**

Human PBMC were isolated from heparinized (10 U/ml) blood by density gradient centrifugation (Lymphoprep, 1114545, 1.077 g/ml, Oslo, Norway). Cells were directly used for subsequent isolation of monocytes and cultured under LPS-free conditions.

For cell enrichment the MACS magnetic separation technique was used (all columns and reagents from Miltenyi Biotec, Bergisch-Gladbach, Germany). For isolation of CD14++ monocytes, PBMC were in a first step depleted of CD16-positive cells. For this, a total of 20 × 10⁶ cells were resuspended in 80 μl of PBS containing 25 μl of Anti-CD16 microbeads (130-045-701). After incubation for 30 min at 4°C cells were washed and resuspended in 1.5 ml PBS and this was loaded onto a LD column (120-000-497) that was positioned in a MidiMACS magnet. Nonadherent cells were recovered and used for enrichment of CD14++ cells. For this, anti-CD14 microbeads (130-050-201) was diluted 1:5 in PBS and added to the cells to a final volume of 100 μl. After incubation for 30 min at 4°C cells were washed and resuspended in 1.5 ml PBS and this was loaded onto a LS column (120-000-475). The column was washed five times with 2 ml PBS each. Cells were recovered from the column by flushing the column five times with 2 ml PBS using a plunger. CD14++ cells were washed and resuspended in supplemented RPMI 1640 medium (mentioned above).

To determine purity of the CD14++ monocytes, a sample was stained with fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibody (My4-FITC, 6603511, Coulter, Krefeld, Germany) and phycoerythrin (PE)-labeled anti-CD16 antibody (Leu11c-PE, Becton-Dickinson, Heidelberg, Germany) and measured by flow cytometry. CD14++ monocytes with a purity of 92% or higher were used.

For generation of MDM, CD14++-monocytes were cultivated in a low-attachment 24-well plate (3473, Costar, Wiesbaden) and cultured for 5 days with 100 ng/ml M-CSF (rhM-CSF, kindly provided by Genetics Institute, Cambridge, Massachusetts, USA).

**Sputum induction and processing**

Sputum macrophages were collected from healthy human volunteers and from patients with COPD after informed consent. The subjects were instructed to mouthwash with water. Sputum induction was performed by stepwise inhalations for 10 min each with increasing concentrations of sodium chloride (0.9%, 3%, and 5% in healthy individuals and 0.9% and 3% in COPD patients), nebulized by an ultrasonic nebulizer (Multisonic LS 290, Schill, Probstzella, Germany). After inhalation individuals were encouraged to cough deeply. Sputum was coughed into petri dishes (d = 13.5 cm) and processed immediately.

To homogenize the sputum samples by cleavage of disulphide bonds of mucin glycoproteins, four volume parts of sputolysin reagent (560000, Calbiochem, Bad Soden, Germany) containing 6.5 mM dithiothreitol and 100 mM phosphate buffer (pH 7.0) were added. After vortexing briefly, the mixture was incubated at 37°C and vortexed every 10 min until the sputum was homogenized, in total no longer than 50 min. The sputum samples were diluted with 1 volume PBS (Gibco, Karlsruhe, Germany) and filtered consecutively through a 100 μm and a 40 μm mesh filter (352360 and 352340, Becton Dickinson, Heidelberg, Germany). Cells were then pelleted by centrifugation for 10 min at 400 × g and 4°C and resuspended in 3 ml PBS. 10 μl packed erythrocytes and 50 μl RosetteSep Human Monocyte Enrichment Cocktail (15068, CellSystems, St. Katharinen, Germany) were added, which crosslinks unwanted cells to multiple red blood cells, forming immunorosettes. After 20 min incubation at room temperature the sample was layered on the top of lymphoprep and centrifuged 30 min at 800 × g. The

| Number | Sex | Age | Disease and Stage |
|--------|-----|-----|------------------|
| normal healthy | 23 | 12 × m, 11 × f | 42.8 ± 15.4 | n.a. |
| healthy smokers | 6 | 4 × m, 2 × f | 54.5 ± 10.6 | n.a. |
| COPD patients | 12 | 10 × m, 2 × f | 63.9 ± 10.5 | 4 × II, 3 × III, 5 × IV (Gold) |
| others (brush biopsies) | 7 | 5 × m, 1 × f, 1 × anonymized* | 65.8 ± 11.7* | adenocarcinoma, MALT-lymphoma, UIP, sarcoidosis (Gold II), COPD (Gold III)* |

(*: samples from one patient who underwent brush biopsy was anonymized; f: female; m: male; MALT: mucosa-associated lymphoid tissue; n.a.: not applicable; UIP: usual interstitial pneumonia).
enriched macrophages were harvested from the plasma interface.

**Brush biopsy of airway epithelial cells**

Patients who underwent diagnostic bronchoscopy were brush biopsied with a sterile single-sheated nylon cytology brush (BC-202D-5010, Olympus EndoTherapy, Hamburg, Germany). Four consecutive brushings from an intrabronchial area were taken from the proximal part of one of the main bronchi. Epithelial cells were harvested from the brush by agitating it in 5 ml of RPMI 1640 medium. After centrifugation cells were lysed in TRI reagent (T-9424, Sigma, Taufkirchen, Germany) and RT-PCR analysis was performed (see below).

**Treatment of cells**

For stimulation with lipopolysaccharide (LPS; L-6261, Sigma, Taukirchen, Germany) blood cells were incubated with 10 ng/ml, sputum macrophages with 1 μg/ml for 3 h each, or remained untreated as control. For particle-stimulation with ultrafine P90 (14 nm in diameter, Degussa, Frankfurt, Germany) and fine TiO₂ (220 nm in diameter, Degussa, Frankfurt, Germany), cells were incubated for 3 h with 32 μg/ml (each particle) or remained untreated as control. For particle concentration experiments CD14++ monocytes were incubated with ultrafine P90 for 3 h at doses of 0.32 μg/ml - 1 mg/ml and 37°C. Using the trypan blue exclusion assay, cell viability was not found to be altered by ultrafine P90-treatment at any particle concentration used herein. For time course experiments cells were incubated for different periods of time (0.5 to 20 h) with P90 (32 μg/ml). For stimulation with BaP (12780, Fluka, Taufkirchen, Germany) a 1:50 dilution of BaP-containing liposomes was used. For preparation of liposomes di-oleyl-phosphoserine (OOPS) and palmitoyloleyl-phosphocholine (POPC) were dissolved at an OOPS:POPC ratio of 0.43 in chloroform. BaP in chloroform was added at different concentrations. Liposomes were generated by evaporating organic solvent followed by adding aqueous buffer. Resulting liposomes can be stored up to 4 weeks at 4°C.

Experiments were performed at least in triplicates, using primary cells from different donors, or cell lines from different passage numbers.

**Total RNA isolation and RT-PCR**

Polymerase chain reaction (PCR) was performed according to the method of Wang and colleagues [42]. Total RNA was extracted from cells by using TRI Reagent (T-9424, Sigma, Taufkirchen, Germany) according to the manufacturer's instruction. In brief cells were lysed in 200 μl TRI Reagent and 15 μg tRNA (109517, Roche, Mannheim, Germany) as carrier were added per sample. After isolation, the RNA was reverse transcribed with oligo(dT) as primer.

Using the LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction, semi-quantitative PCR were performed with the following primers:

- **CYP1A1;** 5' primer: 5'-TCT TTC TCT TCC TGG CTA TCG CT-3'
- 3' primer: 5'-CTG TCT CTT CCC TTC ACT CITG
- **CYP1B1;** 5' primer: 5'-TGA TGG ACG CCT TTA TCC TCT C-3'
- 3' primer: 5'-CAT AAA GGA AGG CCA GGA CAT A-3'
- **MPO;** 5' primer: 5'-TGC GTACCC AGT TCA GGA AGC-3'
- 3' primer: 5'-CCA GGT TCA ATG CAG GAA GTG T-3'
- **α-enolase;** 5' primer: 5'-GTG AGG AAG AAA CTG AAC GTC A-3'
- 3' primer: 5'-TGA AGG ACT TGT ACA GGT CAG-3'

3 μl of cDNA were used for amplification in the SYBR Green format using the LightCycler-FastStart DNA Master SYBR Green I kit from Roche (2239264, Mannheim, Germany). For PCR analysis, the LightCycler system offers the advantage of fast and real-time measurement of fluorescent signals during amplification. The SYBR Green dye binds specifically to the minor groove of double stranded DNA. Fluorescence intensity is measured after each amplification cycle. During PCR, a doubling of template molecules occurs in each cycle only during the log-linear phase. Melting curves have been performed after amplification to ensure that primer dimers did not contribute to the fluorescence intensity of the specific PCR-product. Amplificates were run out on a 2% agarose gel and bands were observed on the expected molecular weight. As an internal control the housekeeping gene **α-enolase** was amplified.

For analyzing the LightCycler data all samples (tested gene and housekeeping gene) are processed in the LightCycler software with the same settings (e.g. same thresholds). Cycle number of the housekeeping gene was subtracted from the corresponding housekeeping gene and its absolute value was subsequently calculated to the power of 2. Genes with a higher cycle number than the corresponding housekeeping gene were plotted to the negative scale.

**Western blot**

For Western blot analysis the microsomal fraction was prepared. After a 32 h incubation with and without 32 μg P90/ml Calu-3 cells were harvested and the pellet was resuspended in 4 volumes of hypo-osmolar buffer A (10...
mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂) in presence of a protease inhibitor mixture consisting of 10 μg/ml aprotinin (A-6279, Sigma), 1 mM PMSF (P-7626, Sigma), 40 μg/ml leupeptin-propionyl (L-3402, Sigma), 20 μg/ml leupeptin-acetate (L-2023, Sigma), 20 μg/ml antipain (A-6191, Sigma), 20 μg/ml pepstatin A (P-4265, Sigma), 400 μM ALLN (A-6185, Sigma), and 2 mM DTT (19474, Merck). The lysates were incubated for 10 min on ice prior to sonication (8 x 10 sec) and then centrifuged at 3,000 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at first at 12,000 × g for 12 min at 4 °C and then at 25,000 × g for 2 h at 4 °C to yield the microsomal fraction. The microsomal pellet was solubilized in 30 μl of buffer D (20 mM HEPES pH 7.9, 20% Glycerol, 0.1 M KCl, 0.05 mM EDTA) in presence of a protease inhibitor mixture and 0.1% SDS and then stored at -80 °C.

Protein concentrations were determined using Bradford reagent. 10 μg of protein were resolved on a 4-12% Novex bis-tris gel (NP0329BOX, Invitrogen, Karlsruhe) and transferred to Hybond-N membranes (LC2001, Invitrogen, Karlsruhe) and membranes then were exposed to Hyperfilm™ECL (RPN3103, Amersham). The antibody was detected using the ECL kit (RPN2106, Amersham). A, Alpha Diagnostic) overnight and anti actin (A-2066, Sigma) and membranes then were exposed to Hyperfilm™ECL (RPN3103, Amersham).

Densitometric analysis was performed using NIH ImageJ software (Version 1.39u, NIH, Bethesda, MD, USA).

**Statistics**
For statistical analysis of the data, we used the Student’s T-test. Results were considered significant if p < 0.05.

**Abbreviations**
BaP: benzo[a]pyrene; COPD: chronic obstructive pulmonary disease; CYP: Cytochrome P450; f: fine; h: hour; LPS: lipopolysaccharide; MDM: monocyte-derived macrophage; P90: Printex 90; PAH: polycyclic aromatic hydrocarbons; PBMC: peripheral blood mononuclear cells; ROS: reactive oxygen species; uf: ultrafine

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
CE was substantially involved in conducting the experiments and drafting the manuscript. MF, FS, AS, and K-WS were in involved in conducting the experiments. LZH is one of the project leaders and was involved in the design of the study. TH is the main project leader, involved in the design of the study, in conducting experiments, and in drafting the manuscript.

All authors read and approved the final manuscript.

**Acknowledgements**
We thank Dr. Wolfgang Kreyling for providing us with ultrafine P90 as well as fine and ultrafine TiO₂ particles.

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