Diesel exhaust particles increase LPS-stimulated COX-2 expression and PGE_2 production in human monocytes

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Abstract: Little is known about health effects of ultrafine particles (UFP) found in ambient air, but much of their action may be on cells of the lung, including cells of the monocyte/macrophage lineage. We have analyzed the effects of diesel exhaust particles (DEP; SRM1650a) on human monocytes in vitro. DEP, on their own, had little effect on cyclooxygenase (COX)-2 gene expression in the Mono Mac 6 cell line. However, when cells were preincubated with DEP for 1 h, then stimulation with the Toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) induced an up-to four-fold-higher production of COX-2 mRNA with an average twofold increase. This costimulatory effect of DEP led to enhanced production of COX-2 protein and to increased release of prostaglandin E_2 (PGE_2). The effect was specific in that tumor necrosis factor gene expression was not enhanced by DEP costimulation. Furthermore, costimulation with the TLR2 ligand Pam3Cys also led to enhanced COX-2 mRNA. DEP and LPS showed similar effects on COX-2 mRNA in primary blood mononuclear cells, in highly purified CD14-positive monocytes, and in monocyte-derived macrophages. Our data suggest that UFP such as DEP may exert anti-inflammatory effects mediated by enhanced PGE_2 production. *J. Leukoc. Biol.* 75: 856–864; 2004.

Key Words: *cyclooxygenase 2 · DEP · inflammation · LPS · Pam3Cys · Mono Mac 6*

INTRODUCTION

Recent epidemiological studies have associated ultrafine particulate matter (PM) in the ambient air with health effects, which include respiratory and cardiovascular effects [1–6]. Several animal and cellular studies point out that fine and ultrafine particles (UFP) have the potential to trigger inflammatory mechanisms. Influx of inflammatory cells [7], expression of inflammatory cytokines [8–10], and release of eicosanoids [11, 12] were reported to be enhanced during exposure to various aerosols. In current concepts dealing with the effects of environmental agents, oxidant-dependent mechanisms are considered to play a major role. Several authors found increased expression of heme oxygenase-1 and interleukin (IL)-8 as well as activation of the c-Jun N-terminal kinase cascade by diesel exhaust particle (DEP) extracts [10, 13, 14]. Up-regulation of the heme oxygenase-1 indicates activation of protective mechanisms to cope with an increased oxidant generation, leading to a redox imbalance and oxidative stress [13]. A long-term activation of inflammatory pathways by ambient aerosols might contribute to a chronic imbalance of the homeostasis in target tissues, such as the airways and the alveolar structures, which may finally lead to irreversible tissue damages. In this context, it is of interest to investigate whether such particles may also activate mechanisms that are anti-inflammatory.

In a recent report, we have analyzed the impact of elemental carbon UFP and titanium oxide (TiO_2) UFP on the phospholipid metabolism in canine alveolar macrophages (AM) [11]. We found during short-time exposure (1 h) with low-particle concentrations a significant production of prostaglandin E_2 (PGE_2) and at markedly higher particle concentrations, a significant release of leukotriene B_4 (LTB_4). The increase of these mediators was preceded by liberation of arachidonic acid (AA) via a phospholipase A_2 (PLA_2)-dependent mechanism. It has been proposed that PGE_2 release by UFP may counterbalance proinflammatory mechanisms, such as the respiratory burst activity, and thereby limit excessive inflammatory response by cells of the innate immune system. PGE_2 is known to suppress cell-mediated, immune responses but to enhance humoral immune responses [15, 16].

We now have asked whether a prolonged time of cell exposure (>1 h) to UFP might induce enzymes involved in synthesis of lipid mediators. Therefore, the present study was designed to investigate the effect of DEP in Mono Mac 6 (MM6) cells on the inducible cyclooxygenase (COX)-2 as a key enzyme in PGE_2 synthesis. Our data demonstrate that DEP exhibits a costimulatory effect on the induction of COX-2

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mRNA and COX-2 protein synthesis by ligands of Toll-like receptor (TLR)2 and TLR4 in MM6 cells. By contrast, there was no costimulatory effect of DEP on the expression of tumor necrosis factor (TNF) after stimulation with lipopolysaccharide (LPS), indicating a selective effect on inducible COX-2.

MATERIALS AND METHODS

Cells and culture conditions

The MM6 cell line [17] was cultured in 24-well plates (#3524, Costar, Wiesbaden, Germany). Tissue culture tubes (#163160, Greiner, Frickenhausen, Germany) were used for exposure experiments. Cell density was 1×10^6 MM6 cells in 1 ml culture medium.

Culture medium consisted of RPMI 1640 (#F1415, Biochrom, Berlin, Germany) supplemented with L-glutamine 2 mM (#25030-024, Gibco, Karlsruhe, Germany), penicillin 200 U/ml, streptomycin 200 μg/ml (#15140-114, Gibco), nonessential amino acids 1–2× (#11140-35, Gibco), and oxalacetic acid, sodium pyruvate, and insulin supplement 10 ml, for 1 L (#O-5003, Sigma, Taufkirchen, Germany). To avoid any inadvertent LPS contamination, we used a culture medium that was filtered through a Gambro ultrafilter U 2000 (#1N50316001, Gambro, Planegg, Germany) to remove LPS [17]. Fetal calf serum (FCS; 10%) was added that had been pretested for low levels of LPS.

For in vitro culture, peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood obtained from healthy volunteers by density gradient separation. Cells were adjusted to 1×10^6 per ml in RPMI 1640 with 10% FCS supplemented with L-glutamine and penicillin/streptomycin and incubated in tissue culture tubes.

Isolation of $CD14^{++}$ monocytes and generation of monocyte-derived macrophages (MDM)

For isolation of $CD14^{++}$ monocytes, PBMC were in a first step depleted of CD16-positive cells. For this, a total of 20×10^6 cells were resuspended in 100 μl phosphate-buffered saline (PBS) containing 25 μl anti-CD16 microbeads (#130-045-701, Miltenyi Biotec, Bergisch-Gladbach, Germany). 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, Miltenyi Biotech), which was positioned in a MidiMACS magnet (#130-042-302, Miltenyi Biotech). Nonadherent cells were recovered and used for enrichment of $CD14^{++}$ cells. For this, anti-CD14 microbeads (#130-050-201, Miltenyi Biotech) were 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 0.5 ml PBS, and this was loaded onto a MS column (#120-000-472, Miltenyi Biotech), which was positioned in a MiniMACS magnet (#130-042-102, Miltenyi Biotech). The column was washed four times with 500 μl PBS each. Cells were recovered from the column by pressing 2 ml PBS through the column for four times. $CD14^{++}$ cells were washed and resuspended in supplemented RPMI-1640 medium (mentioned above) in a final concentration of 1×10^6 in 1 ml culture medium.

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; #332779, Becton Dickinson, Heidelberg, Germany) and was measured by fluorescence-activated cell sorter (FACS). $CD14^{++}$ monocytes with a purity of 96% or higher were used.

For generation of MDM, $CD14^{++}$ monocytes were cultivated in RPMI-1640 medium supplemented as mentioned above. Additionally, 2% human serum was added. Cells were incubated in a low-attachment, 24-well plate (#3473, Costar) and cultured for 5 days. To determine maturation of macrophages, a sample was analyzed by FACS. The mean value of the forward scatter of freshly isolated $CD14^{++}$ monocytes was 91.77 ± 9.65; after culture, forward scatter was increased to 160.17 ± 5.69 (n=3; ±SD; $P<0.05$).

Experimental set-up

If not indicated otherwise, MM6 cells were precultured in the tissue culture tubes for 1 h at 37°C and 5% CO_2. In a set of four tubes, cells in tube three and four were pretreated with DEP [SRM1650a from National Institute of Standards and Technology (NIST), Gaithersburg, MD] up to 32 μg/ml final concentration. After 1 h, LPS (#L-6261, Sigma) was added in tube two and four to a final concentration up to 1 μg/ml, and cells were incubated for further 2 or 4 h, respectively. Tube one was left untreated as negative control, and tube two was used as DEP control.

Preparation of DEP suspension

A stock concentration of DEP in culture medium was vortexed five times for 10 s each and subsequently sonicated for 5 min (Sonorex RK52, Bandelin, Berlin, Germany). This procedure was repeated three to four times.

Total RNA isolation and reverse transcriptase-polymerase chain reaction (PCR)

PCR was performed according to the method of Wang and colleagues [18]. Total RNA was extracted from MM6 cells by using RNAClean (#RC200, Hybaid, Teddington, UK), according to the manufacturer's instruction. In brief, 2×10^4 cells were lysed in 200 μl RNAClean and 15 μg tRNA, as carriers 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, quantitative PCR was performed using the following primers: COX-2 5′ primer [19]: 5′-GCT TTT TAC CTT TGA CAC CC-3′, 3′ primer: 5′-CTG CTC AAC ACC GGA ATT TT-3′; α-enolase 5′ primer [19]: 5′-GTT AGC AAG AAA CTG AAC GTC ACA-3′, 3′primer: 5′-TGA AGG ACT TGT ACA GGT CAG-3′; 3 μl cDNA was used for amplification in the SYBR Green format using the LightCycler–FastStart DNA Master SYBR Green I kit from Roche Diagnostics (#2239264). For quantitative PCR, 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 each amplification to ensure that primer dimers did not contribute to the fluorescence intensity of the specific PCR product. Amplificates were run out on a gel, and bands were observed on the expected molecular weight. As an internal control, the housekeeping gene α-enolase was amplified.

Western blot

Cell proteins (3 μg/lane) were separated on Novex 4–12% bis–tris gels (Invitrogen, Karlsruhe, Germany) and were transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (#RPN2020D, Amersham, Braunschweig, Germany) by electroblotting. Membranes were reacted with 1:1000 dilution of antibody against human COX-2 (mouse anti-human, #804-112-C050, Alexis, Grünberg, Germany). After reaction with goat anti-mouse immunoglobulin G peroxidase conjugate (#A-4416, Sigma) in a 1:2000 dilution, blots were incubated with ECL reagent (#RPN2106, Amersham) and exposed to Hyperfilm ECL (#RPN3103, Amersham).

PGE_2 enzyme immunoassay (EIA)

For sensitive measurement of PGE_2, we determined intracellular levels [20, 21]. Therefore, 5×10^5 MM6 cells were collected after incubation and were centrifuged (400 *g* for 10 min). The cell pellets, resuspended in PBS, pH 7.4, were immediately deproteinized by adding eightfold volume of 90% methanol solution containing 0.5 mM EDTA (#E-6758, Sigma) and 1 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (#56516, Fluka, Taufkirchen, Germany), pH 7.4. The methanolic suspensions were stored at −40°C for 24 h followed by centrifugation at 10,000 *g* for 20 min at 0°C to remove proteins. The methanolic supernatants were stored at −40°C for another 24 h and again, were centrifuged. Aliquots of the obtained supernatants were dried in a vacuum centrifuge and used for determination of PGE_2 by the PGE_2 EIA, which was performed according to the instructions of the manufacturer (Cayman, Ann Arbor, MI). Using commercial PGE_2 added to control cells, the recovery of PGE_2 was determined to be more than 95%.

Statistics

For statistical analysis of the data, we used the Student's *t*-test.

RESULTS

Effect of DEP, LPS, and DEP/LPS exposure on COX-2 mRNA expression

To quantitate COX-2 mRNA levels, we used LightCycler analysis allowing real-time monitoring of cDNA synthesis. MM6 cells were preincubated with DEP (32 μg/ml) for 1 h followed by stimulation with LPS (1 μg/ml) for an additional 2 h. In **Figure 1A**, expression of COX-2 is shown. Quantification of the fluorescent signals generated during the log-linear phase provides accurate information about the starting concentration of the COX-2 transcripts. The measurements indicate higher levels of COX-2 mRNA produced after treatment with DEP/LPS (crossing point of 19) than after LPS (21) or DEP (25) in comparison with unstimulated cells (25.3). These results indicate a minimal increase of COX-2 transcripts for DEP-treated cells versus control cells and a 73.5-fold increase for DEP/LPS-treated cells versus a 20-fold increase of LPS-treated cells compared with the control. Hence, DEP/LPS led to an ~3.6-fold costimulation of COX-2 mRNA when compared with LPS alone. Amplification of the α-enolase housekeeping gene led to nearly identical amplification curves for the MM6 cultures (Fig. 1B). In an average of four experiments, DEP alone had no effect on COX-2 mRNA expression, but it enhanced LPS-induced levels from 17-fold to 36-fold, which is in a range between factor 1.5 and 4 (**Fig. 2**).

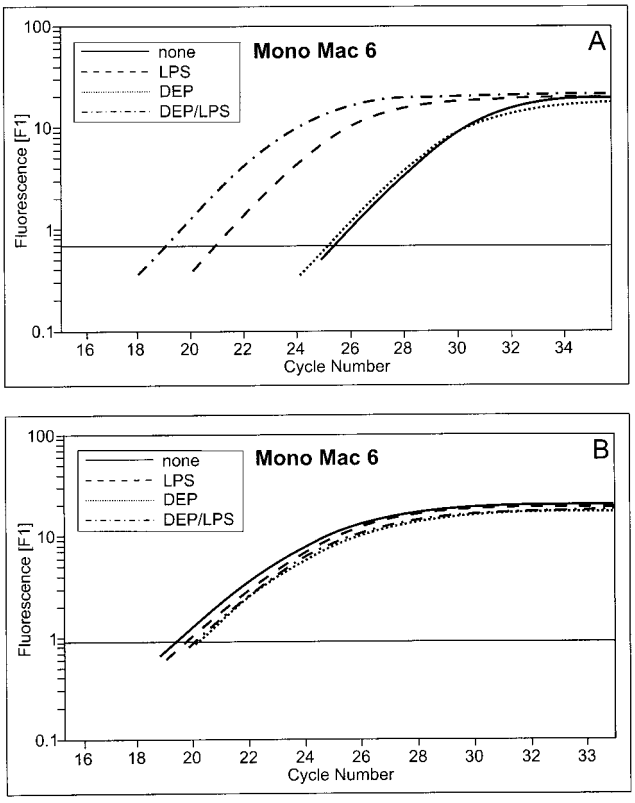

Fig. 1. Effect of DEP and LPS on COX-2 mRNA levels in MM6 cells. Cells were incubated with DEP (32 μg/ml) for 1 h and remained untreated or were costimulated with LPS (1 μg/ml) alone or in combination with DEP for an additional 2 h. Relative COX-2 mRNA levels (A) and α-enolase as a housekeeping gene (B) were analyzed in LightCycler. Shown is a representative experiment out of four.

We also studied time-dependence of COX-2 expression in DEP-stimulated MM6 cells. These studies showed that cells pretreated for 1 h with DEP and then stimulated with LPS for 4 h showed an average 95-fold increase compared with 70-fold increase for LPS alone, indicating that the costimulatory effect of DEP on mRNA was less pronounced at this point in time (data not shown). Also, the DEP-mediated enhancement was seen at lower doses of LPS (2.1-fold at 100 ng/ml and 1.6-fold at 1 ng/ml) and at lower DEP doses (1.85-fold at 10 and 1.5-fold at 1 μg/ml).

Complementary to the effect of DEP on COX-2 mRNA expression, we studied the effect of ultrafine TiO_2, which, alone, did not alter COX-2 mRNA levels (0.9±0.1) compared with untreated cells. LPS and TiO_2/LPS increased COX-2 mRNA levels in the same way (17.6-fold±6.3 and 19-fold±8.1, respectively; n=3; ±SD), indicating no costimulatory effect of TiO_2.

Effect of DEP, LPS, and DEP/LPS exposure on COX-2 expression at protein level

Next, we analyzed the effect of DEP on COX-2 protein. After 2 h incubation, a clearly higher COX-2 protein level can be detected in DEP/LPS-stimulated cells compared with cells stimulated with LPS only (**Fig. 3A**). This effect is more pronounced after 4 h stimulation with LPS. DEP alone shows no significant effect on COX-2 protein expression.

Analysis of Western blots by densitometry showed that stimulation of MM6 cells with LPS for 2 h leads to an increase of COX-2 protein compared with unstimulated cells, and pretreatment with DEP before LPS stimulation leads to an enhanced increase that is 1.6-fold higher than that of LPS alone (Fig. 3B). The synergistic effect between DEP and LPS on the COX-2 protein level after 4 h stimulation with LPS is even stronger. At this point in time, DEP/LPS costimulation results in a 2.5-fold higher COX-2 protein expression than with LPS stimulation alone.

Effect of DEP, LPS, and DEP/LPS exposure on PGE_2 concentration

Downstream to COX-2 protein expression, we investigated the PGE_2 levels in MM6 cells with EIA (**Fig. 4**). Here again, DEP alone had no effect on PGE_2 levels in supernatants, but the LPS-induced PGE_2 level was increased after costimulation by factor 1.7 ($P<0.05$).

Effect of DEP, LPS, and DEP/LPS exposure on TNF and macrophage-inflammatory protein-1β (MIP-1β) mRNA expression

To investigate the specifity of the DEP/LPS synergism on COX-2 mRNA expression, we analyzed TNF mRNA expression in MM6 cells. As shown in **Figure 5**, MM6 cells stimulated with LPS, without and with DEP pretreatment (32 μg/ml), show the same level of TNF mRNA expression. Also, DEP alone does not significantly influence TNF mRNA expression. We additionally investigated the effect of DEP of mRNA expression of the chemokine MIP-1β. In MM6 cells, LPS alone

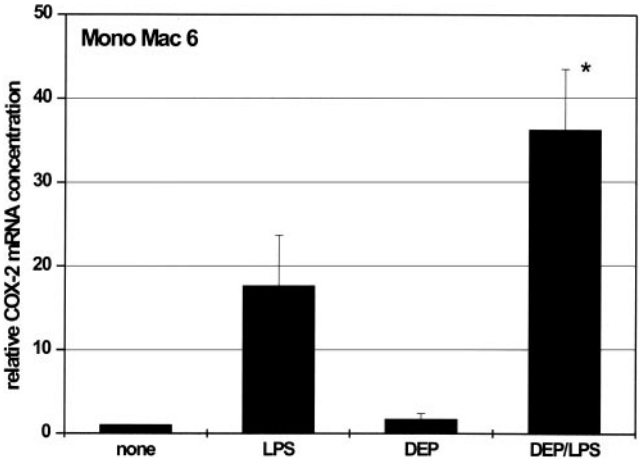

Fig. 2. Effect of DEP and LPS on COX-2 mRNA levels in MM6 cells. Cells were incubated for 1 h with DEP (32 μg/ml) and then remained untreated or were stimulated with LPS (1 μg/ml) alone or in combination with DEP, respectively for an additional 2 h (n=4; ±SD; *, $P<0.05$). Baseline is untreated cells (none) and was set as 1.

induced MIP-1β mRNA 2260-fold ± 725 (n=3; ±SD; $P<0.05$), and after DEP pretreatment (1 h) and subsequent LPS stimulation (2 h), cells responded with a reduced mRNA induction of 780-fold ± 260 (n=3; ±SD; $P<0.05$) compared with untreated cells (data not shown).

Effect of DEP/Pam3Cys exposure on COX-2 mRNA expression

As shown above, DEP shows a cooperative effect on COX-2 induction when LPS stimulates MM6 cells. We were also interested to see whether stimulation of MM6 cells via the TLR2 is enhanced by DEP (**Fig. 6**). After stimulation with Pam3Cys (1 μg/ml) as a ligand of TLR2, we observed a 50-fold increase of COX-2 mRNA expression compared with untreated cells. However, pretreatment with DEP and subsequent stimulation with Pam3Cys resulted in a more than 80-fold increase of COX-2 mRNA expression. Hence, DEP will induce a 1.6-fold enhancement of the TLR2-mediated stimulation of COX-2. Sole treatment with DEP caused a 2.6-fold increase of the low-level COX-2 transcript seen in unstimulated cells.

Effect of DEP, LPS, and DEP/LPS exposure on COX-2 mRNA expression in primary cells (PBMC, $CD14^{++}$ monocytes, and MDM)

To test whether the effect of combined incubation of DEP and LPS on COX-2 mRNA can also be seen in primary cells, freshly isolated PBMC cells, $CD14^{++}$ monocytes, purified from PBMC by magnetic cell sorter (MACS) separation, and MDM were investigated.

In the average of three experiments with PBMC cells, DEP alone led to a twofold increase of COX-2 mRNA. LPS alone increased COX-2 mRNA levels 17-fold, and this LPS-induced level was enhanced after preincubation with DEP by factor 2.1 (range, 2.0–2.4; **Fig. 7**). In $CD14^{++}$ monocytes (n=3), DEP alone had almost no effect on COX-2 mRNA, whereas LPS alone increased COX-2 mRNA sixfold. Compared with LPS alone, LPS with DEP pretreatment enhanced mRNA levels for COX-2 by factor 3.2 (range, 2.1–3.8; **Fig. 8**). In MDM (n=3), DEP alone had no effect on COX-2 mRNA, and LPS alone increased COX-2 mRNA level 16-fold. Compared with the LPS response alone, the costimulatory effect of DEP/LPS stimulation increased COX-2 mRNA levels by the factor 2 (range, 1.5–2.5; **Fig. 9**).

DISCUSSION

This study addressed the question whether abiotic, environmental agents such as DEP modulate the inflammatory poten-

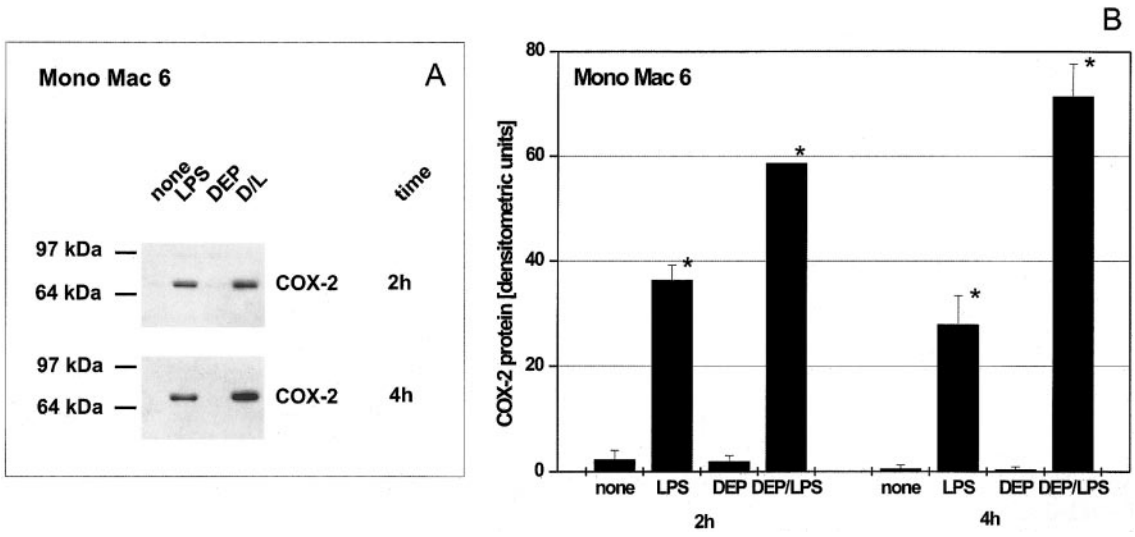

Fig. 3. Western blot analysis of COX-2 protein levels in MM6 cells after incubation with DEP and LPS. Cells were treated with DEP (32 μg/ml) for 1 h and subsequently stimulated with LPS (1 μg/ml) alone or in combination with DEP (D/L) or remained untreated (none) for an additional 2 and 4 h. Shown is a representative Western blot analysis (A). (B) Protein bands were densitometrically analyzed with ImageJ analysis software (Version 1.29, National Institutes of Health, Bethesda, MD; n=3; ±SD; *, $P<0.01$).

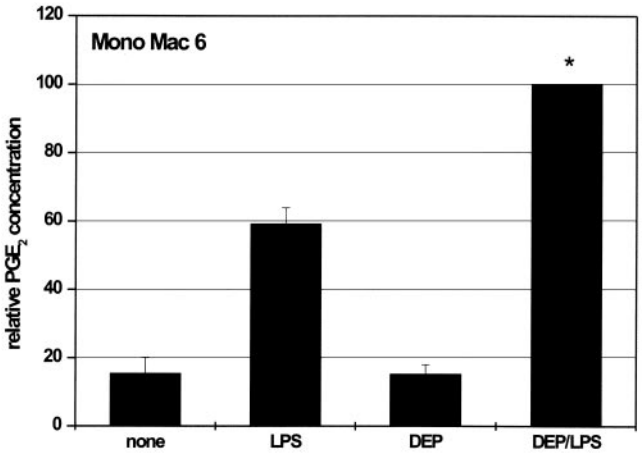

Fig. 4. Relative PGE_2 protein levels secreted by MM6 cells after stimulation with DEP and LPS. Cells were incubated for 1 h with DEP (32 μg/ml) and then remained untreated or were stimulated with LPS (1 μg/ml) alone or in combination with DEP, respectively, for another 2 h. PGE_2 secretion from DEP/LPS-stimulated cells was set as 100% and corresponds to 224 ± 172 pg per 0.5 Mio cells and per ml (n=3; ±SD; *, $P<0.05$).

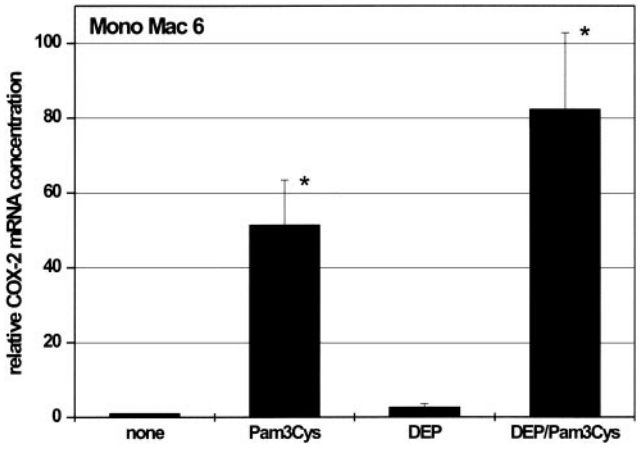

Fig. 6. Effect of DEP and Pam3Cys on COX-2 mRNA levels in MM6 cells. Cells were incubated for 1 h with DEP (32 μg/ml) and then remained untreated or were stimulated with Pam3Cys (1 μg/ml) alone or in combination with DEP, respectively, for another 2 h (n=4; ±SD; *, $P<0.05$). Baseline is untreated cells (none) and was set as 1.

tial of monocytic cells as part of the innate immunity. The rationale for this concept arose from epidemiological findings, suggesting that PM in the ambient air, particularly the ultrafine fraction, causes health effects. Beck-Speier et al. [11, 20] have recently shown that UFP of elemental carbon or TiO_2 induces the rapid release of PGs and LTBs from canine AM. In the present study, we investigated in MM6 cells the expression of COX-2 as the key enzyme of PG synthesis in response to DEP in the absence and presence of bacterial stimuli.

The highest concentration of DEP in the incubation medium in this in vitro study was 32 μg/ml. Looking at urban sites with a high traffic density, this particle concentration appears to be a relevant dose. The range of average concentration of CO, black carbon, total particle number, and mass concentration at 30 m distance down-wind from the source was 1.7–2.2 ppm, 3.4–10.0 $\mu g/m^3$, 1.3–2.0 × 10(5)/cm^3, and 30.2–64.6 $\mu g/m^3$, respectively [22]. Therefore, the particle mass taken up by inhalation within 24 h can accumulate up to 500–2000 μg, and most of it will be deposited in the alveolar region. Assuming 40–50 ml total volume of the human alveolar lining fluid obtained by extrapolation of data from rats (80 μl; ref. [23]) and rabbits (1200 μl; ref. [24]), the accumulative particle concentration after a 24-h deposition ranges between 10 and 50 μg per ml lining fluid. Furthermore, enhanced deposition of particles has been reported at the alveolar duct bifurcations [25, 26], which can lead to an uneven deposition pattern in the lung periphery, including hot spots with extremely high-particle concentrations at the alveolar duct sites.

In the present in vitro study, exposure of MM6 cells to suspended DEP alone did not increase formation of COX-2

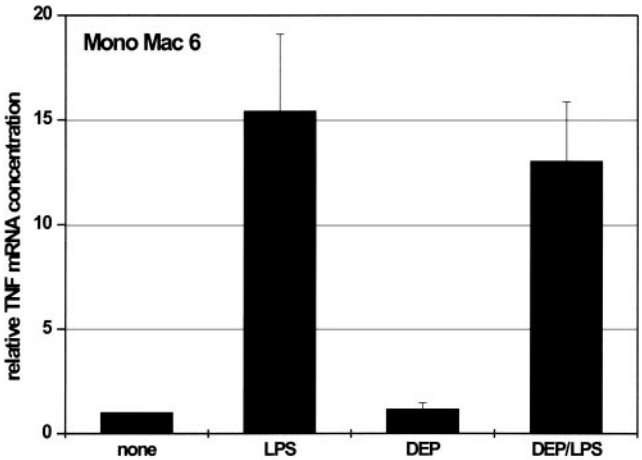

Fig. 5. Effect of DEP plus LPS on relative mRNA levels of TNF in MM6 cells. Cells were incubated with DEP (32 μg/ml) for 1 h. Subsequently, cells remained untreated or were stimulated with LPS (1 μg/ml) alone or in combination with DEP for an additional 2 h (n=4; ±SD). Baseline is untreated cells (none) and was set as 1.

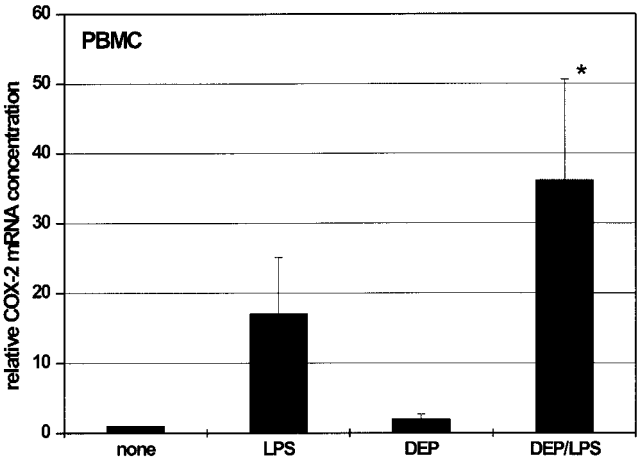

Fig. 7. Effect of DEP and LPS on COX-2 mRNA levels in blood mononuclear cells. PBMC were isolated from blood by density gradient separation, and cells were incubated for 1 h with DEP (32 μg/ml) and then remained untreated or were stimulated with LPS (10 ng/ml) alone or in combination with DEP, respectively, for an additional 2 h (n=3; ±SD; *, $P<0.05$). Baseline is untreated cells (none) and was set as 1.

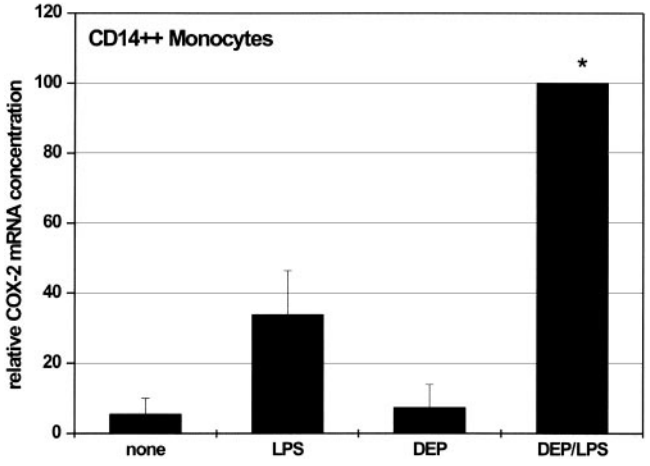

Fig. 8. Effect of DEP and LPS on COX-2 mRNA levels in purified blood monocytes. $CD14^{++}$ monocytes were purified from PBMC by MACS separation, incubated for 1 h with DEP (32 μg/ml), and then remained untreated or were stimulated with LPS (10 ng/ml) alone or in combination with DEP, respectively, for another 2 h (n=3; ±SD; *, $P<0.05$). Baseline is DEP/LPS-treated cells and was set as 100.

mRNA above baseline level. By contrast, stimulation of DEP-pretreated cells with LPS resulted in a clearly enhanced expression of COX-2 transcripts compared with the response to LPS alone. This result suggests a costimulatory effect of DEP on COX-2 induction by TLR4 ligands. Moreover, the absence of a direct effect of DEP on COX-2 induction indicates the absence of significant contaminations by LPS and also shows that DEP per se do not stimulate via TLR4.

In humans, physiologically relevant cells are PBMC, $CD14^{++}$ monocytes highly purified by MACS, and MDM. In the alveoli, there are macrophages that come in contact with inhaled particles. These macrophages have migrated from blood and are similar in properties to monocytes. Using primary human cells such as PBMC (Fig. 7) and human $CD14^{++}$ monocytes highly purified by MACS (Fig. 8), again, we found the same costimulatory effect of DEP after stimulation with LPS, and DEP treatment alone did not elicit a COX-2 transcript response. As macrophages are primary target cells of inhaled particles, we additionally studied the effect of particles on MDM (Fig. 9). These cells also showed a costimulatory effect of DEP. As MDM serve as a model of tissue macrophages, these data suggest that the action of DEP may also be relevant to AM that are exposed to inhaled particles.

The effect of DEP on COX-2 expression could be confirmed by Western blot analysis. The signals on the nitrocellulose membranes (Fig. 3A) reveal two closely adjacent bands, an observation that has already been reported for COX-2 [27]. Results from control and DEP-treated cells without LPS stimulation showed very weak bands, and there was no induction by DEP alone, as evidenced by densitometry of the blots (Fig. 3B). Here, a strong costimulatory effect of DEP on the COX-2 protein is seen after 4 h stimulation with LPS, which compared with the induction of COX-2 mRNA at 2 h, reflects the additional time required for enzyme synthesis. The costimulatory factors of DEP calculated from the DEP/LPS response versus LPS response were similar on the COX-2 mRNA and COX-2 protein level. The fact that the 2 h data point does not reflect the DEP effect to the same extent as the 4 h point is likely a result of DEP action in the form of two optimum curves that are overlapping. As a result, the effect is more pronounced at later time points. The molecular mechanism involved is currently not known.

As expression of COX-2 is one of the limiting steps in the pathway of PGE_2 formation, we analyzed the effect of DEP on PGE_2 production in MM6 cells. Control cells as well as cells pretreated with DEP without LPS stimulation produce only small amounts of PGE_2. This basal, intracellular PGE_2 level is assumed to derive predominantly from the constitutive COX-1 enzyme. However, stimulation of MM6 cells with LPS in the absence of DEP causes a markedly enhanced synthesis of PGE_2, which is twofold higher in cells pretreated with DEP. Here, the effect of DEP on PGE_2 formation after LPS stimulation of MM6 cells is comparable with that on COX-2 expression. Beck-Speier et al. [11, 20] recently reported a rapid release of PGE_2 from canine AM by agglomerates of elemental carbon and TiO_2 UFP and demonstrated dependence of this effect on the particle surface area rather than on their mass concentration. UFP of elemental carbon generated by spark discharge show a specific surface area of 600–700 m^2/g [Brunauer-Emmett-Teller (BET) method] and are ten- to 12-fold more efficient than TiO_2 UFP (50 m^2/g) to release PGE_2 rapidly. It should be mentioned that freshly isolated canine AM produce substantial amounts of PGE_2 released by these particles. This is presumably without induction of COX-2 mRNA and protein, as incubation time of 1 h in the presence of particles is probably too short to allow for substantial protein synthesis. This is in contrast to MM6 cells, which produce only very low amounts of PGE_2 upon exposure to DEP without stimulation with LPS, as mentioned above. The difference observed in the earlier work on canine AM and the present report in human monocytes may be a result of the difference in species, cell type, or particle type used.

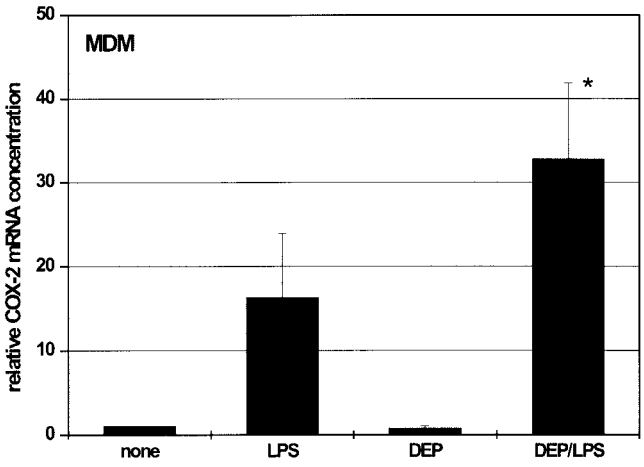

Fig. 9. Effect of DEP and LPS on COX-2 mRNA levels in MDM, which were generated from $CD14^{++}$ monocytes purified from PBMC by MACS separation and subsequent 5-day incubation with 2% human serum. Cells were incubated for 1 h with DEP (32 μg/ml) and then remained untreated or were stimulated with LPS (1 μg/ml) alone or in combination with DEP, respectively, for an additional 2 h (n=3; ±SD; *, $P<0.05$). Baseline is untreated cells (none) and was set as 1.

In a study by Rudra-Ganguly et al. [28], DEP extracts were investigated. Concentrated DEP extracts were shown to inhibit COX-2 enzyme activity, and expression of COX-2 mRNA seems not to be affected. Using nonextracted DEP particles, organic compounds adsorbed to the particle surface may not be bio-available in contrast to extracted compounds and may therefore not contribute to inhibition of the COX-2 enzyme. These authors did not investigate a promoting effect of DEP on and LPS-stimulated COX-2 expression.

According to the provider's certification, DEP from NIST (SRM1650a) exhibit an average surface area of ~100 m^2/g (BET method), suggesting the presence of a considerable fraction of UFP. To decide whether the costimulatory effect on COX-2 expression is specific for DEP, we performed additional experiments with UF-TiO_2. These particles did not show any costimulatory effect with LPS (data not shown), which suggests that UF-DEP promotion of COX-2 expression is selective with regard to particle's physical and chemical composition.

Faced with the obervation that DEP enhance the LPS-stimulated expression of COX-2, we wondered whether ligands of other TLRs would show a cooperative response. We used Pam3Cys for stimulation of the MM6 cells via the TLR2 and found also a costimulatory effect of DEP (Fig. 6). In analogy to TLR4, there is no evidence that DEP interact directly with TLR2 for cell activation. Becker et al. [29] recently reported the involvement of TLR2 and TLR4 in recognition of $PM_{2.5–10}$ taken from ambient air. Gram-positive and Gram-negative bacteria and their degradation products are found in PM of outdoor air in association with inhalable $PM_{2.5–10}$ [30]. Hence, loading environmental particles with bacterial products could explain an involvement of TLRs in the response to the particles. SRM1650a preparation used herein has been produced in the laboratory, and this material is therefore less prone to environmental contamination.

How do unopsonized particles interfere with monocytic cells? In lung macrophages, a role of scavenger-type receptors during uptake of environmental particles has been reported [31]. Another recent study attributed the binding of nonopsonized environmental particulates to the scavenger-type receptor MARCO (macrophage receptor with collagenous structure) by lung macrophages [32]. Whether MARCO or any other cell surface receptor is involved in the costimulation of COX-2 by DEP is currently unclear.

The mechanism, by which DEP particles enhance LPS-induced COX-2 expression is not well understood. One possible explanation might be an increase of the intracellular free Ca^{2+} concentration, which has been shown to be triggered by several particle species. Stone et al. [33] have shown that ultrafine carbon black induces Ca^{2+} influx in MM6 cells. Donaldson et al. [34] reported a rise of intracellular free Ca^{2+} by PM_{10}. Ca^{2+} as a second messenger is generally required for activation of signal transduction pathways, e.g., activation of cytosolic PLA_2 via mitogen activated protein kinases. Beck-Speier et al. [11] reported an increased release of AA from macrophages after treatment with carbon UFP, indicating activation of PLA_2. Choi et al. [35] have shown in microglia cells that influx of Ca^{2+} through store-operated channels is coupled to enhanced COX-2 expression. As the matrix of DEP consists of carbon black-like matter, we conclude that DEP increases the cytosolic Ca^{2+} level and activates PLA_2.

What are the consequences of increased COX-2 expression on exposure to LPS and DEP or other ambient particles? Beck-Speier et al. [11] recently described inhibitory effects of particle-stimulated release of PGE_2 on the respiratory burst activity of monocytic cells/granulocytes. Also, AM from silica-exposed rats have been reported to be preactivated in that they exhibit enhanced prostanoid formation [36]. The authors suggested an anti-inflammatory or immunomodulating role of PGE_2 in silicosis. Other in vivo studies have shown that COX-1 and COX-2 limit allergic inflammation [37], protect from airway hyper-responsiveness [38], and activate anti-inflammatory mechanisms in an animal model of allergic asthma [39]. Such anti-inflammatory effects of PGE_2 may operate via down-regulation of TNF and intercellular adhesion molecule (ICAM) expression [40, 41]. The PGE_2 receptors EP1–4 define the regulatory role of PGE_2. In monocytes, PGE_2 as a major product of COX-2 attenuates inflammatory responses by interaction with the PGE_2 receptors EP2 and EP4 like down-regulation of TNF [40] or IL-18-induced expression of ICAM [41]. In contrast, proinflammatory effects by PGE_2 seem possible when acting via EP1 or EP3. Therefore, the net outcome of a DEP-costimulated PGE_2 cannot be predicted directly.

Looking at proinflammatory cytokines, DEP do not induce expression of TNF in the absence of LPS in MM6 cells, and there is no cooperative effect between DEP and LPS with respect to expression of this cytokine (Fig. 5). This also suggests that there is no LPS contamination in the DEP material from NIST. We therefore postulate that DEP is selective for induction of anti-inflammatory mechanisms and leaves the proinflammatory TNF unaffected. In addition, we have included data on the expression of MIP-1β. When looking at this chemokine, which attracts monocytes and T cells to sites of inflammation, we could observe a down-regulatory effect in DEP-treated cells. These findings suggest that anti-inflammatory mechanisms prevail in response to DEP/LPS.

Of note, Nilson et al. [42] reported on a twofold increase of TNF protein after costimulation of MM6 cells with LPS plus 20 μg DEP/ml. The discrepancy to our finding of unchanged TNF mRNA levels as reported herein is best explained by a post-transcriptional effect of DEP. This could lead, for instance, to higher levels of translation or export of TNF protein. Studies looking at TNF mRNA and protein are required to resolve this issue. In any event, our studies demonstrate specificity at the level of gene expression in that TNF mRNA is unchanged, but COX-2 mRNA is increased by up to factor 4.

Expression of the COX gene has been shown to be controlled at the promoter level [43] and at the level of mRNA stability [44]. Further studies are needed to determine by what mechanisms DEP increase COX-2 mRNA.

Although PGE_2 is produced by the PGE_2 synthase from PGH_2, which is the straight product of the upstream enzymes COX-1 or COX-2, the increase of PGE_2 appears to be closely related to the up-regulation of COX-2.

We propose that DEP costimulation of COX-2 with subsequent production of PGE_2 will exert anti-inflammatory effects in the lung. Such a down-regulation of innate immunity by PGE_2 might render the organism susceptible to pathogens. In

this case, the organisms' ability to defend pathogens might be diminished, which could lead to increased frequency of infection and chronic inflammatory diseases. At this point, it is, however, unclear whether the induction of COX-2 and PGE_2 plays a role in patients with inflammatory responses to inhaled particles, i.e., patients with chronic obstructive pulmonary disease.

Taken together, we demonstrate herein a substantial induction of COX-2 mRNA, protein, and product by pure UFP in human monocytes when costimulated with TLR ligands.

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