Agglomerates of ultrafine particles (UFPs) may cause adverse health effects because of their large surface area. To evaluate physiologic responses of immune cells, we studied whether agglomerates of 77-nm elemental carbon (EC); specific surface area 750 m²/g and 21 nm titanium dioxide (TiO₂) particles (specific surface area 50 m²/g) affect the release of lipid mediators by alveolar macrophages (AMs). After 60-min incubation with 1 μM AUPF-EC (corresponding to 7.5 cm²/particle surface area), canine AMs (1 × 10⁶ cells/mL) released arachidonic acid (AA) and the cyclooxygenase (COX) products prostaglandin E₂ (PGE₂), thromboxane B₂, and 12-hydroxyheptadecatrienoic acid (12-HHT) and the 5-LO products leukotriene B₄ and 5-hydroxyeicosatetraenoic acid (5-HETE). As model particles we used agglomerates of ultrafine particles (UFPs) of elemental carbon (EC) and of titanium dioxide (TiO₂). The biologic effects of these UFPs were compared with those of agglomerates of fine particles (AFPs) of TiO₂.

Materials and Methods

Materials

Phorbol 12-myristate 13-acetate (PMA) and lucigenin were purchased from Sigma (Deisenhofen, Germany); Polymorphprep was from Nycomed (D Sto, Norway); phosphate-buffered saline (PBS) with or without Ca²⁺/Mg²⁺ was from Biochrom (Berlin, Germany); RPMI was from PAA Laboratories (Linz, Austria); fetal calf serum, penicillin, streptomycin and amphotericin were from Life Technologies (Eggenstein, Germany; [¹⁴C]-AA was from Amersham Buchler (Braunschweig, Germany); indomethacin (1-(p-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid) and NS 398 (N-(2-cyclohexyloxy-4-nitrophenyl) methansulphonamide) were from Calbiochem (Bad Soden, Germany); all other chemicals (analytical or high-performance chromatography grade) were from Merck (Darmstadt, Germany).

Particle Characteristics

Ultrathin particles of EC were generated by spark discharging according to the method described by Roth et al. (20). The airborne EC particles had a count median diameter of 77 nm and a specific surface area of 750 ± 150 m²/g (n = 50) as determined by adsorption of nitrogen (21). Ultrathin TiO₂ particles with a diameter of 21 nm and a specific surface area 50 m²/g were purchased from Degussa (Frankfurt, Germany). The fine TiO₂ particles with a diameter of 250 nm and a specific surface area of 6.5 m²/g were

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Address correspondence to I. Beck-Speier, GSF – National Research Center for Environment and Health, Institute for Inhalation Biology, PO Box 1129, D-85758 Neuherberg/Munich, Germany. Telephone: (49) 89-3187-2552. Fax: (49) 89-3187-2400. E-mail: beck-speier@gsf.de

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derived supernatants of different dogs. Asterisks indicate significance of difference between CL of PMA-stimulated PMNs assayed with supernatant of control AMs and super-

percentage of total radioactivity of control cells was 1.97 ± 0.58 [7] for arachidonic acid, 0.43 ± 0.08 [7] for 12-HHT, 0.32 ± 0.11 [7] for 5-HETE, 0.40 ± 0.14 [7] for LTB4, and 0.62 ± 0.21 pre pared by repeated vortexing (5 times for 3 sec) and sonification (1 min) of the suspensions. Despite the vigorous mixing, these particles formed agglomerates as determined by microscopic determination. Therefore, during incubation in suspension, the cells were exposed to AFU Fs and APFs.

Alveolar M acrophages
Canine AMs were harvested by bronchoalveo lar lavage of healthy beagles according to the method of M aier et al. (22). Cells were recovered by centrifugation (400 × g for 20 min) and resuspended in PBS (without Ca2+/Mg2+). Viability was more than 95% as determined by trypan blue exclusion. By microscopic examination of cytospin preparations after May Grünwald Giemsa staining, 85–90% of the cells were identified as AMs.

PMNs
Canine PMNs were isolated from citrate blood samples of healthy beagles by density-gradient centrifugation with Polymorphprep according to Beck-Speier et al. (8). Blood (5 mL) was layered over Polymorphprep (5 mL) diluted with 1.5% (w/v) NaCl. After centrifugation (450 × g for 30 min), separation from lymphocytes/monocytes, and removal of contaminating erythrocytes by hypotonic lysis, a pure population of PMNs (99 ± 1%; n = 3) was resuspended in PBS (without Ca2+/Mg2+) containing 0.1% glucose. Viability was more than 95% as estimated by trypan blue exclusion.

AA Metabolites of AMs
Studies on the AA metabolism in particle-treated AMs were performed as follows: Canine AMs (1 × 106/mL) were preincubated for 2 hr at 37°C in RPMI medium containing penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (2.5 µg/mL), 5% fetal calf serum, and labeled with [14C]-AA (4 kBq/106 cells) in fresh medium for 20 hr. After removal of labeled medium and a preincubation for 30 min at 37°C in PBS, pH 7, containing Ca2+/Mg2+ and 0.1% glucose, AMs were incubated with particles for 60 min at 37°C. Incubation was stopped by cooling to 4°C and [14C]-lipids were extracted from a 1-mL suspension of AMs by addition of 3.75 mL chloroform/methanol (1:2). Phase separation was induced with an additional 1.25 mL of chloroform and 1.25 mL of 0.2% formic acid. The organic phase was collected and the aqueous phase was extracted once again with chloroform (23). After drying the organic phase under nitrogen, the metabolites were dissolved in chloroform and spotted onto high-performance thin-layer chromatography plates (HPTLC) (10 cm × 20 cm; anodurally glass plates; Macherey & Nagel, Düren, Germany). Separation by thin-layer chromatography (TLC) was performed in a solvent system containing ethyl acetate/iso-octane/acetic acid/water (10:5:2:10) according to the method of Krug and Berndt (24). [14C]-AA metabolites were visualized by autoradiography. The Rf values of the metabolites were determined by comparison.
with migration of [14C]-AA and commercial, nonradioactive standards (LTB4, 5-HETE, 12-HHT, TXB2, and PGE2; Paesel and Lore, Hanau, Germany) visualized by phosphomolybdic acid (Macherey & Nagel) on HPTLC plates. The radioactive metabolites were quantified in relation to the total cellular radioactivity by digital autoradiography (Berthold, Wildbad, Germany). Because PGE2 and TXB2 showed identical Rf values under this TLC, they were quantified as a single band.

Respiratory Burst Activity of PMNs by AM-Derived Supernatants

Canine AMs (1 × 10^6/mL) were incubated for 60 min at 37°C in the absence (control) or presence of particles in PBS, pH 7, containing Ca^2+ and 0.1% glucose. If the COX inhibitors either indomethacin (50 µM) or NS 398 (50 µM) were used, cells were preincubated with these inhibitors for 20 min at 37°C followed by incubation with particles. After centrifugation (400 × g for 10 min) the supernatants were isolated and their stimulating potential on respiratory burst activity of PMNs was evaluated by lucigenin-dependent chemiluminescence (CL; [25, 26]). Canine PMNs (1 × 10^6/mL) were preincubated for 10 min at 37°C in a CL analyzer (Autolumat LB 953, Berthold, Wildbad, Germany) in 250 µL PBS, pH 7, containing 0.1% glucose and 0.8 mM lucigenin. Aliquots (50 µL) of AM-derived supernatants were added, and CL signals of PMNs were recorded for 20 min. Subsequently, PMNs were stimulated by PMA (50 ng), and CL was measured again for 20 min at 37°C. AM-derived supernatants of individual canine donors were analyzed at least in duplicate for their effect on respiratory burst activity in PMNs.

Statistical Analysis

Statistical significance was determined by analysis of variance and two-sample t-test (STAT-SAK, Version 2.12 by G.E. Dallal, 1986, Alden, MA, USA). Changes with p ≤ 0.05 were considered significant.

Results

Generation of AA Metabolites by Particle-Induced AMs and Their Effect on Respiratory Burst Activity of PMNs

The effects of AUFP-EC as well as that of AUFP-TiO2 on the formation of AA and the AA-derived COX metabolites 12-HHT and PGE2/TXB2 and the 5-LO metabolites 5-HETE and LTB4 are seen in Figure 1. These metabolites were quantified compared to those of control cells. As shown in Figure 2A, AUFP-EC at 1 µg/mL mass concentration caused a 2-fold increase in generation of AA as well as the COX products 12-HHT and PGE2/TXB2 by AMs compared to control cells. The production of the 5-LO products 5-HETE and LTB4 was enhanced at ≥ 10 µg/mL AUFP-EC, i.e., at 10-fold higher mass concentrations. There was no further increase of eicosanoids at AUFP-EC concentrations of 100 µg/mL or 320 µg/mL (data not shown). In addition we assessed the influence of supernatants derived from particle-treated AMs on PMN defense function. Respiratory burst activity of PMA-stimulated PMNs was reduced by supernatants of AMs incubated with AUFP-EC (Figure 2B). In contrast, there was no suppressive effect on PMN respiratory burst activity by supernatants obtained from control incubations with particles in absence of AMs (data not shown). This suggests the presence of an inhibitory factor produced by AMs exposed to AUFP-EC. To study whether COX metabolites are involved in the inhibitory effect of AM-derived supernatants, we pretreated AMs with the COX inhibitors either indomethacin or NS 398 before particles were added. As seen in Table 1, supernatants from AM incubations with AUFP-EC in the presence of COX inhibitors did not reduce the respiratory burst activity of PMNs.

Table 1. Influence of COX inhibitors on the PMN-inhibiting effect of supernatants derived form AUFP-EC-treated AMs.*

| COX inhibitors used for AM preincubation | Control AMs | AUFP-EC-treated AMs | AUFP-TiO2-treated AMs |
|-----------------------------------------|-------------|---------------------|-----------------------|
| No inhibitor                            | 399 ± 21    | 289 ± 31*           | 257 ± 16*             |
| Indomethacin                            | 408 ± 26    | 396 ± 40            | 404 ± 52              |
| NS 398                                  | 394 ± 38    | 406 ± 49            | 365 ± 45              |

*Supernatants of AMs (1 × 10^6 cells/mL) incubated in the absence (control) or presence of AUFP-EC (10 µg/mL) or AUFP-TiO2 (320 µg/mL) were preincubated with the COX inhibitors either indomethacin (50 µM) or NS 398 (50 µM), respectively, for 10 min before particles were added. As seen in Table 1, supernatants from AM incubations with AUFP-EC in the presence of COX inhibitors did not reduce the respiratory burst activity of PMNs.

Figure 3. AM-derived generation of AA metabolites by AUFP-TiO2 and their effect on the respiratory burst activity of PMNs. (A) [14C]-AA metabolites of AMs (1 × 10^6 cells/mL) incubated with AUFP-TiO2 were quantified according to legend for Figure 2A. The radioactivity as percentage of total radioactivity of control cells was 1.77 ± 0.53 [9] for AA, 0.40 ± 0.14 [9] for 12-HHT, 0.26 ± 0.12 [9] for 5-HETE, 0.36 ± 0.18 [9] for LTB4, and 0.61 ± 0.31 [9] for PGE2/TXB2. (B) Supernatants of AMs incubated in the absence (control) and presence of AUFP-TiO2 were analyzed with PMNs according to legend for Figure 2B. The control value was 2.638 ± 0.469 × 10^6 CL counts during 20 min per 1.5 × 10^6 cells [9].
≥ 32 µg/mL. The supernatants derived from AMs incubated with AUFP–TiO2 reduced respiratory burst activity of PM A-stimulated PMNs (Figure 3B). This decrease of PMN respiratory burst activity was not observed with supernatants derived from AMs treated with COX inhibitors prior to incubation with AUFP–TiO2 (Table 1).

AFP–TiO2 increased AA and the COX products 12-HHT and PGE2/TXB2 in AMs at mass concentrations of 100 µg/mL, and the 5-LO products LTB4 and 5-HETE at mass concentrations of 320 µg/mL (Figure 4A). These are markedly higher-particle mass concentrations than those used for AUFP–TiO2 to produce a similar effect. The supernatants derived from AMs incubated with AFP–TiO2 also reduced respiratory burst activity of PM A-stimulated PMNs (Figure 4B).

Control experiments showed that the viability of AMs after incubation with particles did not change compared to that of control cells (viability about 90 ± 2%, n = 2) as ascertained by trypan blue exclusion. The particles used in our study contained < 0.01% endotoxin as estimated by the Limulus amebocyte lysate assay (Charles River, Sulzfeld, Germany). Because endotoxin in various concentrations up to 10 µg/mL did not influence the formation of eicosanoids in canine AMs, a possible effect of an endotoxin contamination of the particles on the release of eicosanoids can be excluded.

Taken together our results indicate that for each type of particle AA and the COX products PGE2/TXB2 and 12-HHT are generated at distinctly lower particle mass concentrations than the 5-LO products. The mediators present in the supernatants derived from particle-treated AMs are able to suppress PMN defense function. The mediators responsible for inhibition of PMN activity are COX-dependent.

Relationship between Eicosanoid Production and Surface Area of the Particles

The particles used in this study have very different surface areas per mass. To evaluate which parameter of the particles determines the biologic response of AMs, we related the data on the generation of eicosanoids to the mass and surface area of the particles. As shown in Table 2, a 2-fold initial increase of AA, PGE2/TXB2, and 12-HHT by AMs was seen at mass concentrations of 1 µg/mL for AUFP–EC, 10 µg/mL for AUFP–TiO2, and 100 µg/mL for AFP–TiO2. However, these different mass concentrations correspond to very similar surface areas ranging between 5 and 7.5 cm²/mL for the three types of particles. This indicates that the initial release of AA and the COX products PGE2/TXB2 and 12-HHT is induced by similar surface areas of the different types of particles. Table 3 shows a 2-fold initial increase of the 5-LO products LTB4 and 5-HETE by AMs incubated with 10 µg/mL AUFP–EC, 32 µg/mL AUFP–TiO2, and 320 µg/mL. These particle mass concentrations correspond to surface areas ranging between 16 and 75 cm²/mL. Comparing both tables, it is apparent that the 5-LO products are released at higher particle surface areas than AA and the COX products.

Discussion

The present study demonstrates that AUFP–EC, AUFP–TiO2, and AFP–TiO2 induce AMs to release AA as a metabolite of PLA2, PGE2, TXA2 (detected as its inactive metabolite TXB2), and 12-HHT as products of the COX pathway, and LTB4 and 5-HETE as products of the 5-LO pathway. However, the dose-response of the 5-LO pathway to these particles is different from that of the PLA2 and COX pathways. A 2-fold release of PLA2– and COX-related metabolites by AMs occurs at a mass concentration of 1 µg/mL for AUFP–EC but at a 10-fold higher mass concentration for AUFP–TiO2 and 100-fold higher mass concentration for AFP–TiO2 (Table 2). This initial increase of AA, 12-HHT, and PGE2/TXB2 was followed by a further gradual increase up to 300–400% of baseline level for increasing particle mass concentrations (Figures 2A, 3A, 4A). Interestingly, the very different mass concentrations of the three types of particles needed for the initial release of AA and COX products correspond to similar surface areas within a range of 5–7.5 cm²/mL (Table 2). This implies that the surface area rather than the mass concentration of the particles determines the biologic response of AMs to the particles. This conclusion is supported by findings of Oberdörster et al. (5) showing that after instillation in rats the increased pulmonary toxicity of ultratine TiO2 particles was related to their large surface area. Compared to AA and the COX products, the initial increase of the 5-LO-derived metabolites LTB4 and 5-HETE is induced at substantially higher mass concentrations for each type of particle corresponding to surface areas ranging between 16 and 75 cm²/mL (Table 2). This indicates that a higher particle surface area is needed for an initial activation of the 5-LO pathway than for that of PLA2 and the COX pathway. A further increase of particle mass concentrations led to a further increased release of 5-LO products up to 500% of baseline level. At high surface area, AUFP–TiO2 cause a stronger response on...
Effect of ultratine particles on lipid mediators

Table 2. Influence of particle mass concentration and particle surface area on generation of AA and the COX products PGE2/TXB2 and 12-HETE by canine AMs.

| Particle       | AUF-P-EC | AUF-P-TiO2 | AFP-TiO2 |
|----------------|----------|------------|----------|
| Mass/µL        | Surface area/µL | Initial increase | (µL) (%) | (µL) (%) | (µL) (%) |
| AA             | 1.0 ± 0.3 (3)*   | 174 ± 13 (3)* | 172 ± 23 (4)* |
| PGE2/TXB2      | 211 ± 27 (3)*   | 201 ± 47 (3)* | 233 ± 30 (4)* |
| 12-HETE        | 160 ± 30 (3)*   | 200 ± 24 (3)* | 221 ± 41 (3)* |

*Control values are described in legends for Figures 1-3. Asterisks indicate significant differences between control cells and particle-affected cells (p < 0.05).

Table 3. Influence of particle mass concentration and particle surface area on generation of the 5-LO products LTB4 and 5-HETE by canine AMs.

| Particle       | AUF-P-EC | AUF-P-TiO2 | AFP-TiO2 |
|----------------|----------|------------|----------|
| Mass/µL        | Surface area/µL | Initial increase | (µL) (%) | (µL) (%) | (µL) (%) |
| LTB4           | 179 ± 31 (5)*   | 224 ± 71 (7)*  | 289 ± 92 (5)* |
| 5-HETE         | 177 ± 57 (5)*   | 251 ± 63 (5)*  | 286 ± 84 (5)* |

*Control values are described in legends for Figures 1-3. Asterisks indicate significant differences between control cells and particle-affected cells (p < 0.05).

In our study the marked response of the respiratory burst activity of PMNs to the supernatants of AUF-P-treated AMs could be triggered by the mixture of the various lipid mediators present in the supernatants. Inhibition of COX in AUF-P-treated AMs abolished the inhibitory effect of the conditioned supernatants on PMN activity (Table 1). Because COX exists in two isoforms, COX-1 and COX-2, we have used the COX inhibitors indomethacin and NS 398 in concentrations sufficient for each of them to inhibit both isoforms (27). Among the COX-dependent metabolites, PGE2 is known to suppress the respiratory burst activity of PMNs in vitro (18), whereas the 5-LO-dependent mediators LTB4 and 5-HETE are able to activate oxygen radical production (28, 29). The 5-LO products are released from AMs at substantially higher particle surface areas in the incubations than the COX products such as PGE2. Because the inhibitory effect on the PMN activity is seen with AM-derived supernatants obtained by both low and high amounts of particle surface area, we conclude that PGE2 is the predominant mediator for the response of PMNs in this system. A possible stimulating effect of the 5-LO products released at higher particle surface area seems to be suppressed by PGE2.

PGE2 is considered an anti-inflammatory mediator because it suppresses the release of cytokines such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) (16, 17, 30, 31) and downregulates leukotriene synthesis (15). This is consistent with reports documenting that PGE2 upregulates the synthesis of IL-6, which is known to inhibit the production of IL-1β and TNF-α (32), and of IL-10, which plays a crucial role in terminating inflammatory processes (33).

We have shown that PGE2 plays a key regulatory role in the response of AMs to AUF-Ps in vitro. We therefore hypothesize that AM-derived PGE2 downregulates initial inflammatory reactions, which might be induced by inhaled AUF-Ps in vivo to protect the lungs against inflammatory injury and/or to minimize adverse effects. This protective function of PGE2 may be the case for healthy individuals without symptoms of pulmonary and cardiovascular diseases. For patients having chronic pulmonary problems, the protecting role of PGE2 may be overwhelmed by ongoing inflammatory processes including responses of inflammatory cells such as PMNs to inhaled particles.

In several reports an increased production of PGE2 was described during in vitro and in vivo exposure studies with various particulate matter. Mohr et al. (10) reported that AMs of rats exposed to silica with a recovery period of several months showed an increased level of PGE2 and TXB2 that occurred after the elevated TNF-α release declined. These authors concluded that AMs of silica-exposed rats display an enhanced PGE2 production that could serve anti-inflammatory and immunomodulating roles in silicosis. This is in agreement with findings of Englen et al. (34) who reported that silica at low doses caused the release of the COX products PGE2, TXB2, PGF2, and 12-HHT, whereas the metabolites of 5-LO were not produced. However, at higher doses the 5-LO products LTB4 and 5-HETE were also generated accompanied by an increase in cytotoxicity of the silica particles. Kuhn et al. (35) showed that AMs of active coal miners displayed a marked increase in the production of PGE2, TXB2, 5-LO, and TNF-α and a reduction of LTB4 compared to normal volunteers and inactive coal miners. Despite their occupational exposure to coal dust, these miners did not have severe respiratory symptoms, which might be explained by their elevated PGE2 levels protecting the lungs against inflammatory injury. AMs exposed in vitro to freshly fractured coal dust showed elevated levels of PGE2 and TXB2 but normal levels of LTB4, whereas exposure to silica activated the production of all three eicosanoids (36). Taken together, these reports show an activation of the COX pathway with a concomitant release of PGE2 by lower amounts of particulate matter, leading to reduced inflammatory responses. At higher particle mass concentrations the proinflammatory mediator LTB4 is produced in greater generation, which also depends on the chemical composition of the particles. All these findings support our hypothesis that PGE2 protects against adverse effects of low amounts of inhaled particles. Interestingly, Oberdörster et al. (5) have shown that ultratine TiO2 particles phagocytized by AMs and then instilled into rats did not elicit the inflammatory responses caused by the particles alone. This is consistent with a possible protective role of PGE2, which downregulates the inflammatory potency of AMs after uptake of particles.

How AUF-P-EC and AUF-P-TiO2 stimulate AMs to release eicosanoids and which signal transduction mechanisms are involved is not currently known. From a cell-free in vitro system we have obtained evidence that the large surface area of AUF-Ps potently oxidizes methionine to methionine sulfoxide, indicating the formation of oxygen radicals (37). We therefore suggest that during interaction with membranes the oxidative potential of AUF-Ps may initiate radical reactions leading to the activation of PLA2, which hydrolyzes phospholipids with polyunsaturated fatty acids to protect against lipid peroxidation (38). AA generated during hydrolysis of correspondingly substituted phospholipids may be metabolized by COX, initiating the formation of PGE2, and by 5-LO, leading to production of 5-HETE and LTB4. Because these eicosanoids are potent mediators in stimulating inflammatory

cytokine release than AUF-P-EC, which might be due to the different chemical composition of both types of particles.

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cells, the preferred generation of PGE₂ by AUFPs may have the effect of downregulating immune cells and of protecting against increased oxidant burden. Further studies should elucidate these molecular mechanisms in AMs induced by AUFPs, including consequences on gene expression of pro- and anti-inflammatory cytokines and of protective and/or repair enzymes. Because the airway and alveolar epithelia are major targets for inhaled particles, the responses of epithelial cells to AUFPs should also be investigated, including their interactions with AMs.

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