Oxidant Generation and Toxicity of Size-Fractionated Ambient Particles in Human Lung Epithelial Cells

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Exposure to ambient particulate matter (PM) is associated with respiratory and cardiovascular disease and lung cancer. In this study, we used size fractionated PM samples from contrasting locations (three urban sites, one remote background) in the UK with a Sierra-Andersen high volume cascade impactor. The H2O2-dependent oxidant generating capacity of the samples was determined by electron spin resonance with 5,5-dimethyl-1-pyrroline-N-oxide spin trapping. In A549 human lung epithelial cells, we determined the cytotoxicity of samples by using the LDH assay, and the release of pro-inflammatory cytokine levels, including IL-8. Marked contrasts were observed for all endpoints. Remote background PM showed the lowest oxidant potential, was neither cytotoxic nor genotoxic and did not increase IL-8 release. For the other samples, effects were found to depend more on sampling location than on size fraction. PM collected at high-traffic locations generally showed the strongest oxidant capacity and toxicity. Significant correlations were observed between the oxidant generating potential and all toxicological endpoints investigated, which demonstrates that measurement of the oxidant generating potential by ESR represents a sensitive method to estimate the toxic potential of PM.

Introduction

Particulate matter (PM) in ambient air is a mixture of solid and liquid anthropogenic and naturally occurring particles of various sizes and composition (1). Epidemiological studies have found associations between exposure to PM and increasing cardiac and respiratory morbidity and associated mortality (2, 3). Induction of cellular oxidative stress and resulting activation of pro-inflammatory mediators are considered to play a central role in the development of airway diseases like chronic obstructive pulmonary disease (COPD) and asthma (4). Oxidative stress and inflammation are also linked to the formation of DNA strand breaks and oxidative DNA damage by inhaled particles (5). These mechanisms are considered to contribute to carcinogenesis, and thereby may provide some explanation for the observed epidemiological association between PM exposure and lung cancer.

Pulmonary inflammation is characterized by the influx of phagocytes into the lung and up-regulation of cytokines including the potent neutrophil recruiting and activating factor interleukin-8 (IL-8) (6). Measurements of inflammatory cells and pro-inflammatory cytokine levels, including IL-8, in bronchoalveolar lavage fluid have proven to be relevant biomarkers of exposure to PM in human-volunteer studies (7, 8). In vitro studies have demonstrated that PM can up-regulate IL-8 expression in lung epithelial cells (9, 10). Macrophages and neutrophils are major sources of reactive oxygen species (ROS) within the inflamed lung upon their activation. Particle-elicited inflammation and subsequent generation of ROS can lead to oxidative DNA damage, and this pathway is defined as secondary genotoxicity (5). Besides the ability to introduce inflammation, particles may also cause oxidative DNA damage because of their physicochemical properties. Within this so-called primary genotoxicity, surface associated free radicals and transition metals are considered to play a major role (5). Transition metals present in PM like iron cause generation of ROS, specifically hydroxyl-radicals (·OH) via the Fenton-reaction (4, 11).

ROS, including ·OH, are known to cause oxidative lesions to genomic DNA such as the premutagenic adduct 8-hydroxydeoxyguanosine (8-OHdG) (5). Higher rates of 8-OHdG are a well accepted risk factor for the development of cancer. ROS have also been implicated in the ability of PM to activate signaling pathways that lead to activation of inflammatory mediators, including IL-8 (4). Taken together, this indicates that the measurement of the ROS-generating capacity of PM represents a promising method to predict inflammatory and mutagenic effects of these ubiquitous air pollutants (12–14). Electron spin resonance (ESR) is a method that has recently been advanced to monitor the hydroxyl radical (·OH) generation capacity of ambient PM samples (12). Specifically, with this method the H2O2-dependent formation of ·OH is determined by ESR with the aid of the spin-trapping agent 5,5-dimethyl-1-pyrrolin-N-oxide (DMPO). By using this method we could previously show, that the ability of fine PM to generate ·OH is related to their ability to cause oxidative DNA damage in lung epithelial cells (14).

In the present study we used ESR to characterize the ·OH generating capacity of size fractionated PM samples (3–7, 1.5–3, 0.95–1.5, 0.5–0.95, and <0.5 µm), collected in ambient air at four contrasting locations (three urban sites, one remote background). While the chemical analysis of the samples has been described and discussed previously (15) the focus of our present study was to determine the cytotoxic, genotoxic, and inflammatory properties of these well-characterized samples in relation to their ·OH-generating capacity. In A549 human lung epithelial cells, we determined the toxicity of samples by using the LDH assay, and the release of IL-8 by ELISA as a marker of pro-inflammatory action. Moreover, we determined oxidative DNA damage using the formamido-pyrimidine-glycosylase (fpg)-modified comet assay.

Experimental Section

Collection of Particulate Matter by Size-Fractionated Sampling. Size-fractionated samples of ambient PM were
TABLE 1. Characteristics of the Sampling Campaign and Locations

<table>
<thead>
<tr>
<th>location</th>
<th>period</th>
<th>number of PM samples collected</th>
<th>characteristics of sampling location</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>remote background</td>
<td>08/08/2002–28/08/2002</td>
<td>research station Mace Head in Connemara on the west coast of Ireland, the sampling site is located on a beach facing the Atlantic Ocean, all samples represent a rather pure maritime aerosol</td>
</tr>
<tr>
<td>UB</td>
<td>urban background</td>
<td>12/07/2002–28/07/2002</td>
<td>urban background site on the campus of the University of Birmingham. Measurements were made over grassland, 1.5 m above ground level, and with road traffic being distant at least 100 m</td>
</tr>
<tr>
<td>BR</td>
<td>Bristol Road</td>
<td>08/07/2002–17/07/2002</td>
<td>roadside sampling site in Birmingham beside the A38 road, one of the major arteries of radial traffic (22000 vehicles/day)</td>
</tr>
<tr>
<td>QR</td>
<td>Queensway road tunnel</td>
<td>02/07/2002–07/07/2002</td>
<td>road tunnel sampling site inside the Queensway underpass, part of the A38 road</td>
</tr>
</tbody>
</table>

* A more detailed description of the sampling sites has been published by Birmili et al. (15).

collected in 2002 at four contrasting observation sites, ranging from a rural observation site on the west coast of Ireland to three urban sites of varying traffic-influence (urban background, roadside, and road tunnel) in Birmingham, UK. The particle sampling procedure, chemical analysis, and also a discussion of the results as a function of meteorological air masses were described in the former article by Birmili et al. (15). Briefly, ambient PM samples were collected using a Sierra-Andersen high volume cascade impactor. Polytetrafluoroethylene (PTFE) filters were used to collect particles on the impactor plates (size fractions 3–7, 1.5–3, 0.95–1.5, and 0.5–0.95 µm), and as a back-up filter (<0.5 µm). The four sampling locations were chosen to represent rather different situations of PM exposure, and show marked differences with respect to the anthropogenic contributions to the collected PM (15). For the characteristics of the sampling locations and periods see Table 1. For the present investigations we selected a subset of 80 PM samples from all samples that were previously collected on the PTFE, that is, 16 samples per size fraction and 20 samples per sampling location, respectively. All samples used in this study were collected between July 2nd and August 28th, to avoid effects of seasonal variation.

Extraction of Particles from the Filters for Oxidant Measurements and Toxicological Investigations. Samples were extracted from the filters by adding 10 mL of distilled endotoxin-free H2O to each filter. The samples were shaken for 5 min (vortex), then 5 min sonicated and finally shaken again for 5 min. From each obtained suspension, one part was used for the immediate analysis of the oxidant activity of the PM using electron spin resonance (ESR). The remaining part of each suspension was transferred into a 15 mL polystyrene container (Greiner, Germany) and freeze-dried. The extracted particle mass was determined by weighing the containers after extraction. The average variation of all samples (the number is specified in the results section).

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**Analysis of Oxidant Capacity of the PM Samples by Electron Spin Resonance.** Formation of -OH by the collected PM samples was evaluated by ESR as described earlier (12), with minor modifications. Briefly, 100 µL of the freshly prepared particle suspension was mixed with 200 µL of the spin trap 5,5-dimethyl-1-pyroline-N-oxide DMPO (0.05 M in PBS) and 100 µL H2O2 (0.5 M in PBS). The mixture was incubated for 15 min at 37 °C in a shaking water bath and filtered through a 0.1 µm filter (Millipore, U.S.).

**Cell Culture and Treatment of Cells.** Human A549 lung epithelial cells (American Type culture collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 1% L-glutamine (200 mM, Sigma), 1% penicillin/streptomycin (Sigma), and 10% heat inactivated fetal calf serum (FCS, Sigma) at 37 °C and 5% CO2. For experiments, cells were seeded into 96-well plates or 24-well plates at a density of 40 000 cells/cm². After 24 h, cells were synchronized for 16 h in reduced DMEM (0.1% FCS). PM samples were thawed, suspended in complete medium, vortexed for 2 min, sonicated (Sonorex TK52 water-bath; 60 W, 35 kHz) for 10 min and then directly added to the cells at a concentrations of 100 µg/cm² for 4 or 24 h. Concentration and time points used in this study were chosen on the basis of pilot experiments with pooled samples (urban background PM) to obtain sufficient material.

**Determination of Cytotoxicity by the LDH Assay.** Cytotoxicity was measured as the release of lactate dehydrogenase (LDH) by the cytotoxicity detection kit (Roche Diagnostics, Germany). Briefly, after incubation of the test samples for 24 h, cell culture supernatants were collected by centrifugation and used for LDH activity measurement according to the manufacturer’s protocol. Results are expressed as LDH release as % control.

**Determination of the Release of Interleukin-8.** The release of the IL-8 from the A549 cells was analyzed by a commercial ELISA Kit (Sanquin, The Netherlands). After 24 h treatment, cell-free supernatants were collected and analyzed according to the manufacturer’s protocol. Results are expressed as IL-8 release as % control.

**Determination of Strand Breaks and Oxidative DNA Damage by the fpg-Modified Comet Assay.** Oxidative DNA damage induced by the collected PM samples was determined by the fpg-modified comet assay. Because of insufficient amounts of PM, this assay was performed only in a subset of all samples (the number is specified in the results section). After treatment for 4 h with PM suspensions, the A549 cells
were rinsed twice with PBS, detached by trypsinization and immediately suspended in FCS containing culture medium. Cells were centrifuged for 10 min at 400 g, resuspended in medium, and immediately processed for the fpg-comet assay and analyzed as described previously ([6]. As reference samples (included in each electrophoresis run) aliquots of a single batch of frozen RLE cells were used in which oxidative DNA damage was induced by treatment with a photosensitizer plus visible light. The level of oxidative DNA damage for each sample was determined as described in the text. For cytotoxicity determination the LDH assay was used, which indicates loss in cell membrane integrity (shown in Figure 2). PM samples from the remote background site did not yield a significant toxicity (Figure 2). Two-factor ANOVA revealed clear differences in toxicity for the samples collected at the four different locations after correction for particle size fraction (respectively, *P < 0.05, *P < 0.01 and ***P < 0.001 versus RB within the same size fraction). For four out of the five particle fractions (3–7, 1.7–3, 0.95–1.5 μm), the oxidant capacity turned out to be a strong function of the observation site, increasing by roughly 2 orders of magnitude between the remote background and the road tunnel. For the finest particle fraction (<0.5 μm) the trend was reversed, but below statistical significance. When comparing oxidant capacity across the various size fractions, one can also see different trends between different observation sites: At the remote background, oxidant capacity appears to decrease with increasing particle size, while at the roadside and road tunnel sites, the reverse is true.

**Effects of the Samples on Human Lung Epithelial Cells.** For cytotoxicity determination the LDH assay was used, which indicates loss in cell membrane integrity (shown in Figure 2). PM samples from the remote background site did not yield a significant toxicity (Figure 2). Two-factor ANOVA revealed clear differences in toxicity for the samples collected at the four different locations after correction for particle size fraction (respectively, *P < 0.05, *P < 0.01 and ***P < 0.001 for UB, BR, and QR vs RB). Significant differences in toxicity between the five different size fractions upon adjustment for sampling location were not observed. The highest release of LDH was measured for the Bristol Road and Queensway Road tunnel PM.

**Interleukin-8 Release.** Figure 3 shows the IL-8 release from A549 cells after treatment for 24 h with the different PM samples. Because of the large sample-to-sample variation relatively few significant differences in IL-8 release were found when comparing specific size fractionated samples from different locations by one-way ANOVA. Two factor ANOVA revealed a significant IL-8 release from A549 cells treated with the finest particle fraction (<0.5 μm) as compared to the release upon treatment with other size fractions after adjustment for sampling location (P < 0.001). Moreover, IL-8

**Results**

**Oxidant Capacity of the Samples.** Relative intensities of mass-adjusted ·OH generation as measured by ESR are shown in Figure 1. Note that the ESR signals are shown on a logarithmic scale. Marked contrasts were found in the oxidant capacities of samples from both, different particles size fractions and sampling locations. Analysis by two-factor ANOVA revealed that the ·OH generation differed by sampling location, whereas no significant differences were seen for particle size. Urban background (UB), Bristol Road (BR), and Queensway Road tunnel (QR) PM showed significantly increased ESR values compared to the remote background samples after adjustment for the size fraction (P < 0.05, P < 0.001, and P < 0.001, respectively). For out of the five particle fractions (3–7, 1.7–3, 0.95–1.5 μm), the oxidant capacity turned out to be a strong function of the observation site, increasing by roughly 2 orders of magnitude between the remote background and the road tunnel. For the finest particle fraction (<0.5 μm) the trend was reversed, but below statistical significance. When comparing oxidant capacity across the various size fractions, one can also see different trends between different observation sites: At the remote background, oxidant capacity appears to decrease with increasing particle size, while at the roadside and road tunnel sites, the reverse is true.

**Statistical Analysis.** One-way analysis of variance (ANOVA) with Dunnett post hoc comparison was used to test for differences between sampling locations per size fraction (comparing the samples from remote background (RB) to each of the other locations), as well as to compare differences between size-fractions (comparing the <0.5 μm size fraction samples to all other size fractions). Data shown in the graphs represent mean and standard deviations (SD), with level of significance indicated by the number of asterisks, that is, *P < 0.05, and **P < 0.01 and ***P < 0.001. ESR values were analyzed after log-transformation. A subsequent two-factor ANOVA was applied to determine for the respective effects after adjustment for sampling location and particle size fraction categories. Linear regression analysis was used to determine the level of correlation among the different effect parameters. Statistical analyses were performed using SPSS 18.0.
release was also found to differ with sampling location after particle size fraction adjustment ($p < 0.001$ for BR and QR vs RB).

Oxidative DNA Damage. The fpg-modified comet assay was used to determine the induction of oxidative DNA damage in A549 cells after treatment with the collected size-fractionated PM samples. As can be seen in Figure 4, the remote background and urban background samples were found to be less potent in inducing oxidative DNA damage in the A549 cells than the samples from the high-traffic locations (BR and QR). Two-factor ANOVA analysis demonstrated that oxidative DNA damage effects were independent of size fraction but not for sampling site indicating that the location is the prime factor to determine the potential of generating oxidative DNA damage to the cells.

Relation between Oxidant Capacity and Cellular Effects of the Samples. To determine whether measurement of the oxidant capacity of the samples as measured by ESR was related to the observed (geno)toxic and inflammatory responses, linear correlation analyses were performed. Results are shown in Table 2 and Figure 5. When all samples used in this study were considered together, significant correlations were found between the oxidant capacity of the samples and cytotoxicity, IL-8 release as well as oxidative DNA damage induction. The correlations improved if the smallest fraction particles (<0.5 µm) were excluded from analysis. For the particles <0.5 µm no correlation between ESR signals and IL-8 release or oxidative DNA damage was found, whereas a significant inverse relation was observed with the LDH-release (see Table 2). Overall the strongest associations were found between the ·OH generating properties of the samples and their ability to induce oxidative DNA damage (Figure 5). Further significant relationships were found between DNA damage and LDH release, and between DNA damage and IL-8 release, whereas LDH and IL-8 did not show any correlation. Noteworthy, oxidative DNA damage was determined only for a subset of the samples due to insufficient amounts of PM available. However, the correlations between ESR, IL-8 and LDH observed for this subset
Particle faction (but not on particle size, with the exception of the smallest showed that the generation of ESR signals was found. ESR measurements in the present study damage and IL-8 release) a significant correlation to the ESR. For all measured endpoints (cytotoxicity, oxidative DNA importance of the oxidant properties of PM for their effects. \(12, 13\)). Several methods have been introduced to measure the radical generating properties of PM, including electron spin resonance (ESR). Cytotoxic, inflammatory and genotoxic effects in A549 cells were measured as LDH leakage, IL-8 release, and oxidative DNA damage, respectively. In each cell of the table linear correlation coefficients, sample size in brackets, and the level of significance are depicted (ns = not significant).

### Discussion

Induction of cellular oxidative stress, resulting from the formation of free radicals by specific constituents of PM is considered to play a central role in their adverse health effects (4). Several methods have been introduced to measure the 
\(\cdot\)OH generating capacity of PM samples in aqueous suspension and integrates a number of aspects, including redox activity of bound and soluble transition metals as well as the bioavailability of these metals for reaction (13). The data from our present study are in support of the importance of the oxidant properties of PM for their effects. For all measured endpoints (cytotoxicity, oxidative DNA damage and IL-8 release) a significant correlation to the ESR signals was found. ESR measurements in the present study showed that the generation of \(\cdot\)OH depends on the sampling but not on particle size, with the exception of the smallest particle faction (<0.5 \(\mu\)m particles). PM collected at the traffic-influenced locations generally showed a higher oxidant generating capacity than samples of the same particle size fraction collected at places with less or no traffic. These findings are in line with previous publications (14). Importantly however, we did not observe such contrasts for the smallest size fraction (<0.5 \(\mu\)m). For these particles, we even observed a reversal of the general trend in the ESR signals: The \(\cdot\)OH generation by the PM samples from the rural locations was on average at least as strong as that from the samples collected at the high-traffic locations. These findings are likely due to the specific ESR technique and method that we used in this study (12). We suspect that the relatively low signals for the finest particles may be due to \(\cdot\)OH-quenching effects of traffic-derived soot constituents, which are enriched in the smallest particle fraction from the tunnel dust and road dusts relative to the urban background and remote sample. This would also explain for the observation that the ESR signals for the smallest size fraction (<0.5 \(\mu\)m) were highest when collected at remote background and lowest in the high traffic locations. Moreover, it should be emphasized that our ESR method specifically determines the \(\text{H}_2\text{O}_2\)-dependent generation of \(\cdot\)OH: Previously, we demonstrated...
that for our ESR method the transition metal content of the particles, especially iron and copper, is a dominating factor (12). For ultrafine, combustion-derived PM, ROS generation is also known to be associated with their comparatively high amount of polycyclic aromatic carbons (PAH) and related organics through quinone cycling mechanisms (11, 17).

It is nowadays generally accepted that exposure to PM can lead to various adverse health effects and that ROS are playing an important role herein (4, 18). Among the ROS, the ·OH radical is of greatest concern because of its high reactivity and hence its ability to attack crucial cellular macromolecules including the genomic DNA (5). To investigate the ability of the collected PM samples to introduce oxidative DNA damage, we analyzed the A549 cells after treatment with PM by the highly sensitive fpg-comet assay. The results indicate that PM from the immediate vicinity of traffic sources leads to a higher induction of oxidative DNA damage than the particles sampled in the urban or rural background. Since the fpg-comet assay specifically detects lesions occurring from oxidative DNA attack, present results also demonstrate that traffic-related PM is more potent in inducing cellular oxidative stress. For the observed effects of PM, both the chemical composition and surface reactivity are considered to play a dominating role. PM generated from combustion-processes are composed of a poorly soluble core onto which various toxic molecules including metals or PAH can bind and therefore be carried from the environment into the body (5, 19). Both PAH and transition metals are considered to contribute to PM-elicited oxidative stress and DNA damage induction (5). In traffic-rich environments, PAH related compounds are formed upon the burning of traffic engine fuels. Transition metals such as iron or copper within PM samples from locations with a high traffic volume are considered to originate from tires, brakes, and even from the vehicular chassis. The significant presence of transition metals, notably Fe, Cu, Ba, Al, Zn, Mn, and Ni in the urban PM samples under study was confirmed by chemical analysis, as previously published (15). In previous investigations we and others have shown the importance of specific metals in the induction of cellular oxidative stress and DNA damage (4, 7, 14, 17, 20). Interestingly, various metallic constituents that were found to be far more abundant in the PM samples (21). Together, our findings indicate that traffic-derived particles may be particularly harmful to the genomic DNA. Obviously, our current associative findings for remain to be verified for PM in mechanistic studies. Moreover, with regard to the potential implications of current genotoxicity observations for the carcinogenicity of specific PM samples, one should be cautious. The induction of oxidative DNA damage in the A549 cells was also found to correlate to cytotoxicity as determined by LDH release, and therefore it cannot be ruled out entirely that the observed DNA damage may at least in part be an effect secondary to cell death.

In the present study, a contrasting effect on IL-8 release was also observed with the various PM samples. IL-8 is a major pro-inflammatory chemokine, produced by various cell types including lung epithelial cells, macrophages and endothelial cells. It functions as a chemoattractant and recruits neutrophilic granulocytes to the inflammatory site, and therefore is a key parameter in localized inflammation. In our study, the particulate size as well the location had a significant impact on IL-8 production from the A549 human lung epithelial cells. In contrast to the DNA damaging effects of the samples, IL-8 production was not significantly correlated to the cytotoxicity of the samples as determined by the LDH assay. Interestingly, the samples collected at the high-traffic locations showed, rather irrespective of their size, the highest capacity to increase the production of IL-8. As such, these findings are in contrast to previous in vitro and in vivo data from us as well as other investigators where higher cytokine production and pulmonary inflammation were observed upon treatment with coarse PM than with fine PM (22, 23). Importantly however, our current study design allowed for a more detailed evaluation of potential size distribution-dependency of the pro-inflammatory effects of PM. It was therefore interesting to observe that the smallest particles (<0.5 µm fraction) overall gave the strongest IL-8 responses. For coarse and fine PM, soluble transition metals and the content of the endotoxins are considered key components for the development of acute inflammatory processes after treatment with characterized PM samples in vitro (22, 24). We based our treatments on equal mass basis, and therefore the smallest fraction (<0.5 µm) has the largest surface area of all size fractions tested. As such our findings support the general observation that inflammatory effects of small particles are mainly driven by their large surface area (25). However, the observation that the <0.5 µm samples from the rural location were comparatively ineffective in triggering IL-8 release suggests that specific urban/traffic-related constituents (e.g., metals, PAH) (17) are also important for this size fraction. The relative abundance of metals such as Cd, Pb, Mn, Co, Cu, and Zn as measured previously in the ultrafine PM from the traffic-rich environments (15), points toward the pro-inflammatory potency for these specific metals.

We were able to demonstrate that size fractionated PM samples (3–7, 1.5–3, 0.95–1.5, 0.5–0.95, and <0.5 µm) generate hydroxyl radicals in aqueous environment and,
depending on their sampling location, can cause significant cytotoxicity, oxidative DNA damage, and release of the proinflammatory cytokine IL-8 in human lung epithelial cells. Importantly, the effects were found to depend more strongly on the sampling location than on the particle size fraction. For all toxicity parameters, the samples collected at the high-traffic volume locations showed stronger effects than the urban background and rural sample even if considered on an equal mass basis. These aspects should be taken into account in the risk assessment of specific size modes of ambient PM. Moreover, in the present study we observed highly significant correlations between the hydroxyl-radical generating capacities of PM and various end points in human lung epithelial cells that are considered to be relevant markers of their toxicity. This provides further support for the measurement of the oxidant generating potential by ESR as a sensitive tool to estimate potential adverse health effects of environmental particulate matter.

Acknowledgments

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