Formation of reactive oxygen species in rat epithelial cells upon stimulation with fly ash

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Fly ash was used as a model for ambient particulate matter which is under suspicion to cause adverse pulmonary health effects. The fly ash was pre-sized and contained only particles < 20 μm including an ultrafine fraction (< 100 nm) that contributed 31% to the particle number. In our study, we investigated the influence of fly ash on the promotion of early inflammatory reactions like the formation of reactive oxygen species (ROS) in rat lung epithelial cells (RLE-6TN). Furthermore, we determined the formation of nitric oxide (NO). The cells show a clear dose-response relationship concerning the formation of ROS with regard to the mass of particles applied. Lipopolysaccharide (LPS) added as a co-stimulus did not increase the formation of ROS induced by fly ash. Furthermore, in LPS (0·1 μg/ml) and tumour necrosis factor-alpha (TNF-alpha; 1 ng/ml) pre-treated cells no increase in reactive oxygen species comparable to fly ash alone is observable. In presence of the metal chelator, desferrioxamine (DFO), ROS formation can be significantly reduced. Neither fly ash nor LPS induced a significant NO release in RLE-6TN cells.

[Voelkel K, Krug H F and Diabaté S 2003 Formation of reactive oxygen species in rat epithelial cells upon stimulation with fly ash; *J. Biosci*. **28** 51–55]

1. Introduction

Epidemiological studies show that ambient air pollution causes adverse pulmonary health effects, especially in individuals with pre-existing lung diseases, such as, bronchitis, asthma and pneumonia (Schwartz 1994). The particulate fraction of ambient air pollution originating from anthropogenic activities is mainly composed of combustion particles. Industrial combustion is a well studied process and the fly ash produced is often used as a model particle for fine and ultrafine components in toxicological studies (Smith *et al* 1998). In this study, we investigated the effects of fly ash on rat lung epithelial cells. It has been shown that besides alveolar macrophages, which are responsible for clearance of particles from the lung, epithelial cells are capable of internalizing particles, especially the small ones (< 2 μm; Lehnert 1993). They also secrete biological mediators, including cytokines, and express adhesion molecules (Simon and Paine 1995; Papi 1997). In our *in vitro* study, we were especially interested in the effects of fly ash on the promotion of early inflammatory reactions like the formation of reactive oxygen species (ROS) and the formation of nitric oxide (NO) after allowing time for the expression of the inducible nitric oxide synthase (iNOS). In order to mimic the situation of pre-existing lung diseases, we pre-treated the cells with lipopolysaccharide (LPS) or tumour necrosis factor-alpha (TNF-alpha) before fly ash was added to the cells.

Keywords. Desferrioxamine; fly ash; nitric oxide radical; reactive oxygen species; rat lung epithelial cells

Abbreviations used: BSA, Bovine serum albumin; DCF, 2′·7′-dichlorofluorescein; DFO, Desferrioxamine mesylate; DMEM, Dulbecco's Modified Eagle Medium; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; H_2DCF, 2′·7′-dichlorodihydrofluorescein; IFN-gamma, interferon-gamma; iNOS, inducible nitric oxide synthase; MAF98, fly ash used in this study; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein-2; NO, nitric oxide; ROS, reactive oxygen species; TNF-alpha, tumour necrosis factor-alpha; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

LPS mimics the exposure to Gram-negative bacteria and TNF-alpha mimics the presence of activated alveolar macrophages sitting on the alveolar epithelium and releasing TNF-alpha as an early response to endotoxin. To determine the influence of metals adsorbed to the fly ash, we conducted experiments with the specific iron (III) chelator, desferrioxamine.

In this study, we show that besides phagocytic cells such as macrophages and neutrophils which respond to incubation with particles with a respiratory burst, epithelial cells generate ROS after exposure to particles. ROS may have been produced by a reaction of the transition metals, which are constituents of the fly ash, with cellular constituents, for example H_2O_2. This possibility and others are discussed further under § 4.

2. Materials and methods

2.1 *Cell culture*

RLE-6TN epithelial cells (ATCC Rockville, MD, USA) have been immortalized by transfection of rat alveolar type II cells with SV40 DNA (Driscoll *et al* 1995). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% heat inactivated fetal calf serum (FCS; both Gibco, Karlsruhe, Germany), penicillin at 100 units/ml and streptomycin at 100 μg/ml.

2.2 *Preparation of particles*

Fly ash (MAF98) from a commercial municipal waste incinerator was selected as a model particle. It was collected by electrostatic precipitation and had been size-fractionated so that the fly ash contained only particles < 20 μm including an ultrafine fraction (< 100 nm) that contributed 31% to the particle number (Diabaté *et al* 2002). All particle suspensions were freshly prepared in DMEM for nitrite determination or in Hank's balanced salt solution (HBSS; Gibco, Karlsruhe, Germany) for ROS determination and sonicated.

2.3 *Intracellular oxidative stress assay*

The formation of ROS was measured via monitoring the increasing fluorescence of 2′·7′-dichlorofluorescein (DCF) after oxidation of 2′·7′-dichlorodihydrofluorescein (H_2-DCF). The cell-permeant 2′·7′-dichlorodihydrofluorescein (H_2DCF-DA, Molecular Probes, Eugene, OR, USA) enters the cell where intracellular esterases cleave off the diacetate group. The resulting H_2DCF is retained in the cytoplasm as a probe for ROS. Assays were performed using a 96 well flat-bottom plate according to Wan *et al* (1993). The cell density was adjusted to $2{\cdot}5 \times 10^5$/ml and 200 μl of the cell suspension was pipetted into each well. Cells were allowed to attach over night and subsequently washed once with HBSS. Loading with dye (final concentration 50 μM) and fly ash occurred simultaneously and monitoring of the fluorescence at 485 nm ± 10 nm excitation and 530 nm ± 12·5 nm emission wavelengths started immediately afterward for various time points using an automated fluorescence reader (MWG-Biotech AG, Ebersberg, Germany) interfaced with a computer. In the meantime, the plate was incubated in a 37°C humidified incubator gassed with 95% air and 5% CO_2. For comparison, cells were co-exposed to other stimulatory substances like lipopolysaccharide (LPS from *Pseudomonas aeruginosa*, Sigma, Taufkirchen, Germany) and tumour necrosis factor-alpha (TNF-alpha, PharMingen, Heidelberg, Germany) or were pre-treated with those substances for 24 h. When experiments were carried out with the specific iron (III) chelator, desferrioxamine (DFO; Sigma, Taufkirchen, Germany), cells were first loaded with dye for 40 min, subsequently washed with HBSS and then stimulated with the desired substances in the absence or presence of DFO (4 mM). Data were processed with the program Lambda KC4 (Version 2.7, MWG-Biotech AG, Ebersberg, Germany) and then transferred to statistics and graphic programs for further analysis.

For nitrite release tests RLE-6TN were stimulated with fly ash in the absence or presence of LPS (0·1 μg/ml).

2.4 *Nitrite assay*

The formation of the NO radical after exposure to fly ash for 24 h was quantified by the determination of nitrite in the supernatant medium by the Griess method (Green *et al* 1982), and absolute amounts were calculated from a sodium nitrite standard curve using a linear curve fit (OD 550 nm, SOFTmax® PRO software, Molecular Devices).

2.5 *Statistical analysis*

Values are reported as mean ± standard error of the mean (SEM). Statistical analysis was performed in case when experiments were carried out at least in triplicate using Student's *t* test.

3. Results

The fly ash used in this study has been described by Diabaté *et al* (2002). RLE-6TN cells show a clear dose-response relationship concerning the formation of ROS depending on the mass of particles (figure 1). At the subtoxic fly ash concentration of 100 μg/ml, the ROS forma-

tion is increased 2·5-fold in comparison to control after a 2 h exposure. After simultaneous treatment with fly ash and LPS (0·1 μg/ml) as co-stimuli, the formation of ROS is not increased compared to the same fly ash concentration alone (figure 1). For direct comparison, we tried to mimic the situation of pre-existing lung diseases by pre-treating the cells for 24 h with fly ash (100 μg/ml), LPS (0·1 μg/ml) or TNF-alpha (1 ng/ml) and additionally exposing them for 2 h with 200 μg/ml fly ash. Overall, we could observe that 24 h pre-incubation of RLE-6TN did not result in a further increase in the formation of ROS compared to cells not pre-treated (figure 2). In order to investigate the influence of bioavailable transition metals adsorbed to the fly ash, we used the iron (III) chelator, DFO. In fact, we could demonstrate that in the presence of DFO fly ash-induced ROS formation was significantly reduced by 35% compared to cells not treated with DFO (figure 3). Neither fly ash nor LPS alone or in combination induced a significant NO release in RLE-6TN cells. The maximum nitrite concentration of 0·46 ± 0·12 μM determined by the Griess assay was close to the lower detection limit for the assay (figure 4).

4. Discussion

Oxidative stress is defined as a shift in the prooxidant/antioxidant balance in favour of the former (Sies 1985). Enhanced generation of ROS leads to modification of different kinds of biomolecules that are essential for the integrity of cellular structures, including lipids, DNA and proteins. In this study, we could show that rat lung

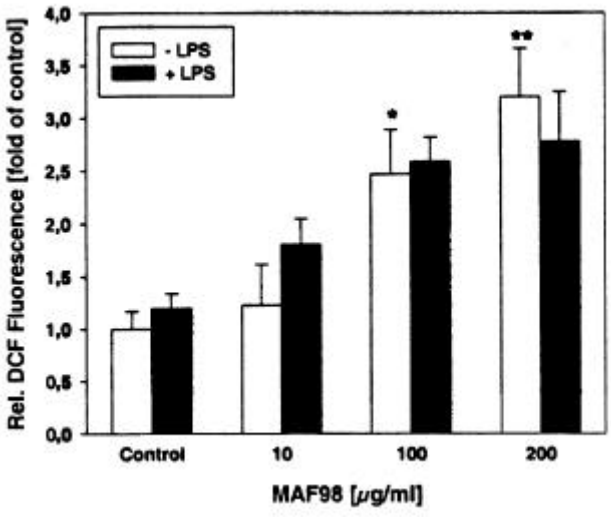

Figure 1. Formation of ROS in RLE-6TN cells after exposure to different concentrations of fly ash (MAF98) in the absence or presence of LPS (0·1 μg/ml). Fluorescence of DCF relative to control was measured after 2 h of exposure. Results are the mean of three experiments ± SEM (each experiment was carried out in triplicate) and are given as multiple of control ($n = 3$; $^{*}P < 0{,}05$; $^{**}P < 0{,}01$ comparing increasing fly ash concentrations to control using Student's t test).

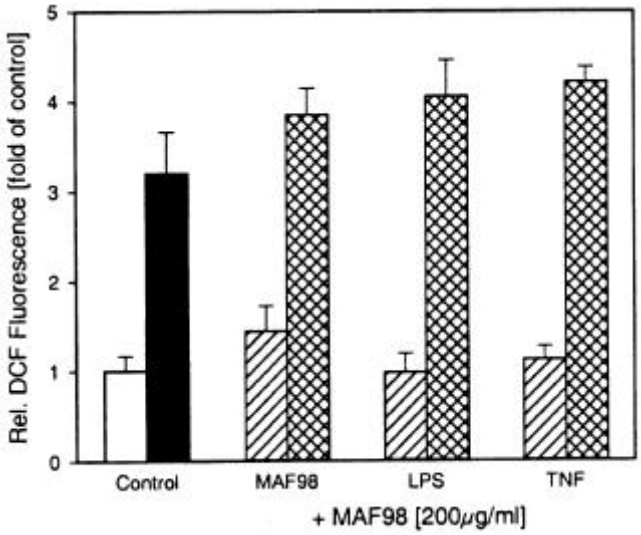

Figure 2. Effect of a 24 h pre-incubation of RLE-6TN cells with certain substances on the formation of ROS induced by fly ash (200 μg/ml). Pre-treatment was carried out with fly ash (MAF98; 100 μg/ml), LPS (0·1 μg/ml), or TNF-alpha (1 ng/ml) for 24 h (hatched bars) and cells were further exposed to fly ash (200 μg/ml) for 2 h (crosshatched bars). Controls were not treated (open bar) or treated with 200 μg/ml MAF98 for 2 h (black bar). Fluorescence values relative to control are means of three experiments ± SEM (each experiment was carried out in triplicate) (no significance was definable).

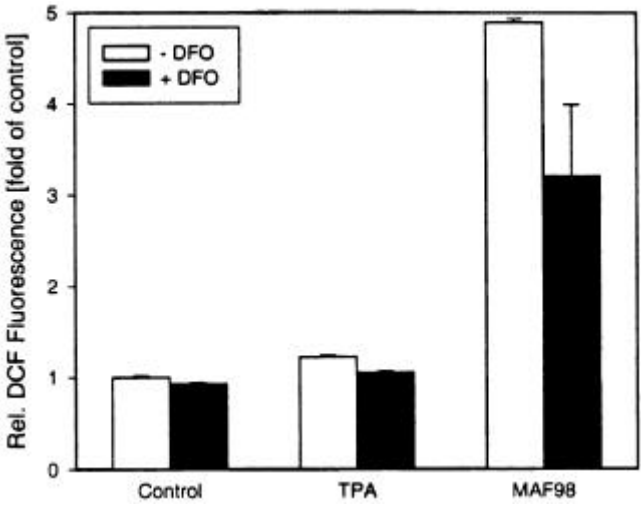

Figure 3. Influence of the iron(III) chelator, desferrioxamine (DFO), on the formation of ROS in RLE-6TN cells. Cells were treated with fly ash (MAF98; 100 μg/ml) or TPA (0·2 μM) for 2 h in the absence or presence of DFO (4 mM). Fluorescence values relative to control are means of two experiments ± SEM (each experiment was carried out in triplicate).

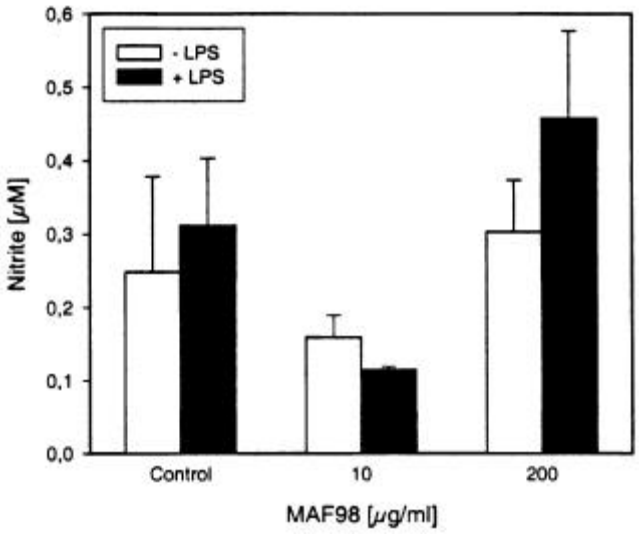

Figure 4. Nitrite concentration in the supernatant medium of RLE-6TN cells after incubation with fly ash for 24 h in the absence (open bars) or presence of 0·1 μg LPS/ml (black bars). Results are the means of two experiments ± SEM (each experiment was carried out in duplicate).

epithelial cells respond to fly ash exposure with the formation of reactive oxygen species in a dose-dependent manner. Experiments that combined the effects of pre-treatment of the cells with LPS or TNF-alpha and subsequent exposure of the cells to fly ash failed to demonstrate a further increase in ROS formation above fly ash alone. A possible source of intracellular oxidants is the mitochondria, which could be placed under stress from the addition of the fly-ash. The resulting leakage of oxidants could then contribute to the dye oxidation seen after fly-ash exposure, making the cells appear to undergo a 'respiratory burst'. In this context we conducted first experiments with inhibitors of the mitochondrial electron transport chain. But one should also take into account that the intracellular oxidation of DCF comes from other sources rather than a respiratory burst produced by the cells. It could also be a chemical (Fenton-like) reaction, in which transition metals in the fly ash could interact with cellular components, for example H_2O_2 to produce oxidants (e.g. hydroxyl radical). The findings that ROS formation is reduced by 35% in the presence of the metal chelating ligand, desferrioxamine, supports this hypothesis**,** indicating that free metals are necessary for some of the oxidation to occur. In this context, Fe^{3+} is a potential candidate because it is bound to desferrioxamine with a stability constant near 10^{31}. The hint that bioavailable transition metals seem to be involved in the induction of cytotoxic and inflammatory effects has already been shown in studies with other fly-ash species like residual oil fly ash (ROFA, Dreher *et al* 1997) or coal fly ash (CFA, van Maanen *et al* 1999).

In contrast to these findings, reactive nitrogen species are poorly detectable after fly-ash exposure. Moreover, simultaneous exposure with fly-ash and LPS as an inductor of the inducible nitric oxide synthase (iNOS) in macrophages had only weak effects on NO production. Although epithelial cells are not known to express the membrane-bound CD14, they can be activated by LPS, namely with the aid of the soluble form of CD14 (Frey *et al* 1993). Human endothelial and epithelial cells require serum proteins for endotoxin stimulation because lipopolysaccharide binding protein (LBP) and the soluble form of CD14 are plasma components (Pugin *et al* 1993). Furthermore, we have found that this is also true for rat lung epithelial cells. These cells, when grown in medium containing bovine serum albumin (BSA), showed no MIP-2 (macrophage inflammatory protein-2) release after LPS stimulation in contrast to cells grown in medium containing fetal calf serum (FCS) (data not shown). In addition, it has been published that in contrast to rat cell lines primary cultures of rat type II pneumocytes showed increased expression of iNOS and enhanced production of NO after treatment with interferon-gamma (IFN-gamma) or a mix of IFN-gamma and TNF-alpha (Punjabi *et al* 1994).

Our data presented here demonstrate that exposure to particulate matter from industrial combustion processes triggers the immediate formation of intracellular oxidants in rat lung epithelial cells that may result from a chemical reaction, intracellular stress or a combination of both. The identification of the specific mechanisms are under investigation. Furthermore the special role of particle size and its impact on these early inflammatory reactions are further analyzed.

Acknowledgements

We thank David Thiele for technical assistance and the Ministry of Environment and Traffic, Baden-Württemberg, for financial support.

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