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Mechanisms of genotoxicity of inhalable particles: *in vitro* & *in vivo* investigations

Inaugural-Dissertation

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Anton Wessels
aus Papenburg

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aus dem Institut für umweltmedizinische Forschung an
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Tag der mündlichen Prüfung:

"Alle Ding' sind Gift
und nichts ohn' Gift;
allein die Dosis macht,
das ein Ding' kein Gift ist."

Paracelsus (1493-1541)

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List of abbreviations

AM	Alveolar macrophage
AP-1	Activator protein-1
APE-1	Apurinic/apyrimidinic endonuclease-1
a.u.	Arbitrary unit
BAL	Bronchoalveolar lavage
BALF	BAL fluid
BER	Base excision repair
BSA	Bovine serum albumine
b.w.	Body weight
cDNA	complementary DNA
CNP	Carbon nanoparticles
COPD	Chronic Obstructive Pulmonary Disease
d_{ae}	Aerodynamic diameter
DEP	Diesel exhaust particles
DMEM	Dulbecco's modified Eagle's medium
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DQ 12	Dörentrup Quartz "ground product nr. 12"
EC	Elemental carbon
EDTA	Ethylenediaminetetraacetic acid
e.g	For example
ESR	Electron spin resonance
EPA	Environmental Protection Agency
EU	European Union
FCS	Foetal calf serum
fpg	Formamido-pyrimidine-DNA-glycosylase G
g	Gram, gravity
GSH	Glutathione
GV-SOLAS	Guidelines provides for the Society for Laboratory Animals Science

h	Hour
HBSS	Hanks' Buffered Salt Solution
HO-1	Heme Oxygenase-1
H_2O	Water
H_2O_2	Hydrogen peroxid
HPLC	High performance liquid chromatography
IARC	International Agency Research on Cancer
IL-8	Interleukin-8
iNOS	inducible Nitric Oxide Synthase
IVCAB	In vivo comet assay buffer
l	Liter
LDH	Lactate dehydrogenase
LMP	Low melting point
m	Meter, milli
M	Molar
MGG	May-Grünwald / Giemsa
min	Minute
MPO	Myeloperoxidase
mRNA	messenger RNA
n	Nano
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NFKB	Nuclear factor kappa B
NO	Nitric oxid
NOX	NADPH oxidase
OC	Organic carbon
Ogg-1	8-oxoguanine glycosylase
OH	Hydroxyl radical
PAH	Polyaromatic hydrocarbons
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PM	Particulate matter
PMA	Phorbol-12-myristate-13-acetate
PMNs	Polymorphonuclear neutrophils

POL β	Polymerase ß
PTFE	Polytetrafluoroethylene
qRT-PCR	quantitative Real Time -PCR
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RTLS	Respiratory tract lining fluid
SD	Standard deviations
SEM	Standard error of mean
SOD	Superoxide dismutase
TNF-α	Tumour necrosis factor- α
UFP	Ultrafine particle
vs.	Versus
WHO	World Health Organisation
XRCC1	X-ray Repair Complementing defective repair
γ-GCS	Gamma-glutamylcysteine synthetase
8-OHdG	8-hydroxydeoxyguanosine
µ	Micro
℃	Degree celsius

Chapter 1

1. Introduction

The human lung has a large surface area (40-120 m²) and is exposed to between 10,000 and 20,000 litre of ambient air each day (1). Thus, air pollutants easily have access to the human body. According to an assessment from the World Health Organisation (WHO), more than 2.4 million premature deaths each year are related to the effects of urban outdoor- and indoor air pollution (2).

That particulate matter (PM) can lead to adverse health effects is described since the late middle ages. Agricola (1494-1555) reported the association between mining and illness. Paracelsus (1493-1541) related the work in mines to lung diseases (3). Historically, particle research focussed on high occupational exposures during work. Coal miners and workers in coking industries were exposed in former days to dust-concentrations up to 40 mg/m^3, whereas nowadays in mining an exposure situation of 2-3 mg/m^3 can occur. Nowadays, general workplace limit values are introduced. Until the mid 20th century no great effort was undertaken to monitor/restrict air pollution for protection of the general population. This thinking mainly changed, after the London smog episode in 1952. It has been estimated that the smog caused 12,000 deaths and is one of history's most important air pollution episodes in terms of its impact on science, public perception of air pollution, and government regulation (4). Since this time air pollution is also of public concern and many epidemiological studies have been performed. Dockery and co-workers in 1993 showed that per 10 µg/m^3 increase in annual PM concentration mortality is increased by 1.4% and respiratory diseases by 4 % (5). These investigations and observations led to a European directive (1999/30/EG), which regulates the ambient PM_{10} concentration. Since 1st January 2005 the daily limit value is 50 µg/m^3, which is not to be exceeded more than 35 times per calendar year. The acceptable annual mean of PM_{10} is 40 µg/m^3. In 2008 the directive was updated and provides additional standards for $PM_{2.5}$ (2008/50/EC).

In the following chapter the composition of particles, reactive oxygen species (ROS) generation and particle-related health effects are described to understand the need of limit values and to provide a useful basis for further reading of this thesis.

1.1 Ambient particulate matter

1.1.1 Particle size distribution

Particulate matter (PM) is a heterogeneous mixture of solid and liquid particles present in the air. The size distribution of particles in the urban air is conventionally characterised by three parameters according to the aerodynamic diameter of the particles (figure 1).

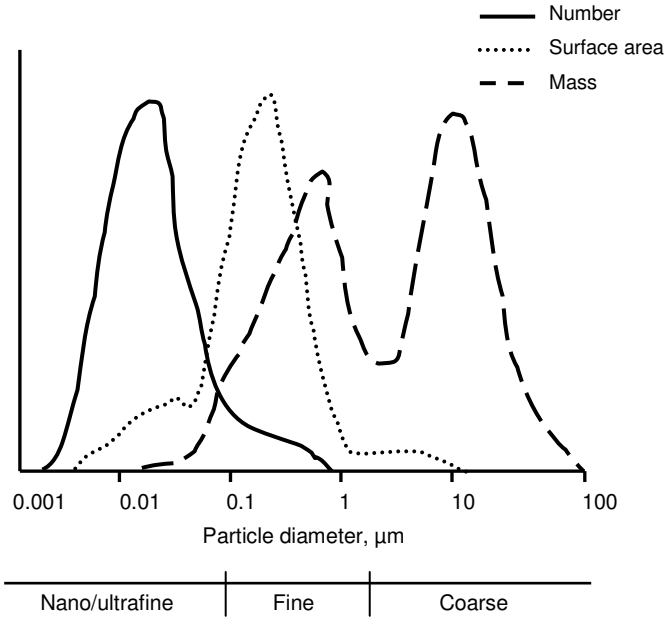

Figure 1. Particle size distribution of ambient PM by three characteristics: mass, surface area and number (adapted from Finlayson-Pitts, 2000).
The black curve represents the distribution pattern by number of particles and includes mainly ultrafine particles (< 0.1 µm). Distribution by surface area constitutes fine particles from 0.1 -1.0 µm (dotted curve). Mass distribution is both fine and coarse particles from 0.1-100 µm (dashed curve). ultrafine particle: < 0.1 µm, fine particle: < 2.5 µm, coarse: > 2.5 µm

The aerodynamic diameter is calculated as the diameter of a unit density (1 g/cm^3) sphere having the same settling speed as the specified particle of whatever shape and density. Thus, particles with identical inertial properties have the same aerodynamic diameter irrespective of their actual size, shapes or densities (6). For standard classification of PM the American EPA (Environmental Protection Agency, 1997) introduced a “national air quality standard for particulate matter” and defined for ambient particulate matter different size-fractions according to the aerodynamic diameter (table 1), which have been taken over by the EU (1999) and WHO (2000).

Fraction	*Size*
PM_{10}	< 10 µm
coarse	Particles between $PM_{2.5}$ and PM_{10}
$PM_{2.5}$ (fine)	< 2.5 µm
$PM_{0.1}$ (ultrafine/nano)	< 0.1 µm

Table 1. PM standards.

PM_{10} include all other size fraction and these particles are small enough to reach the thorax region of the lung. $PM_{2.5}$ includes the ultrafine fraction and particles are able to reach the alveolar region (7, 8).

1.1.2 Sources & composition

PM is highly heterogeneous in the composition of components. Generally, one can distinguish between natural and anthropogenic sources.

The smallest < 0.1 µm particles have been emitted to the air as primary particles mainly by combustion sources and include elemental carbon from diesel vehicles and metals derived from engine wear. Ultrafine particles (UFP) have a limited lifespan of minutes to hours since they aggregate into larger particles (secondary particles) between 0.1 and 1 µm. Within this accumulation mode most UFP become gradually enlarged. This mode constitutes fine particles, which are mainly formed by coagulation and by fossil fuel combustion. In addition, secondary organic- or inorganic particles are also contributing to this size fraction. Coarse particles mainly originate from natural sources such as dust and sea salt, but also from mechanical processes like mining or wearing of tires and brakes. The coarse and fine fractions contribute very much to the total mass of urban particles, but have little impact on the total surface area. In contrast, the ultrafine particles are the most numerous but weigh the least. In addition, because of their high number they strongly contribute to the total surface area of ambient air particles. Another interesting point is the travel distance, which can be covered by the different fractions. The UFP and coarse particles rarely travel more than 1-10 km whereas the accumulation mode can travel several hundred kilometres, depending on atmospheric conditions (9).

Overall, most of the occurring particles are of natural origin. However, it has to be considered, that in urban environments most of the occurring PM derives from

anthropogenic sources. These include secondary organic and inorganic material, traffic or other combustion-derived particles and resuspended road dust.

	Ultrafine	Accumulation + Fine	Coarse
Sources	Combustion	Combustion	Mechanically generated
	Engine Exhaust	Biomass burning	Dust
	Nucleation	Industry	Sea salt
	Gas to particle conversion	Agglomeration	Resuspension
	Natural emissions	Secondary aerosols	Biogenic material
		Resuspension	(e.g. pollen)
Main components	EC, OC, SO_4^{2-}, metals	NH_4^+, SO_4^{2-}, NO_3^-, EC, OC, metals	Si, Al, Ca, Fe, Na, Cl, OC

Table 2. Main sources and compositions of the three size fractions of particulate matter. EC = elemental carbon, OC = organic carbon. (Adapted from Sillanpää 2006, 97)

1.1.3 Particle deposition

The main route of entry for ambient PM to the body is via inhalation. The deposition of particles within the lung depends on particle size, breathing patterns and lung structure (10). Three mechanisms are important for the deposition of particles. (a) Sedimentation by gravitational forces acting on particles > 0.5 µm, (b) impaction caused by their inertial mass acting on particles > 1.5 µm, and (3) diffusional motion of particles <0.5 µm. The respiratory tract can be divided in three regions based on structure, size, and function, in particular the nasal-pharyngal, tracheobronchial and the alveolar region. Depending on the particle size and mass, the deposition rate varies between these regions. Particles larger than 3 µm and smaller than 0.003 µm tend to deposit in the nasal-pharyngeal region, whereas particles between 0.003 µm and 3 µm reach the alveolar region, with a maximum deposition (about 50 %) for 20-30 nm particles (figure 2).

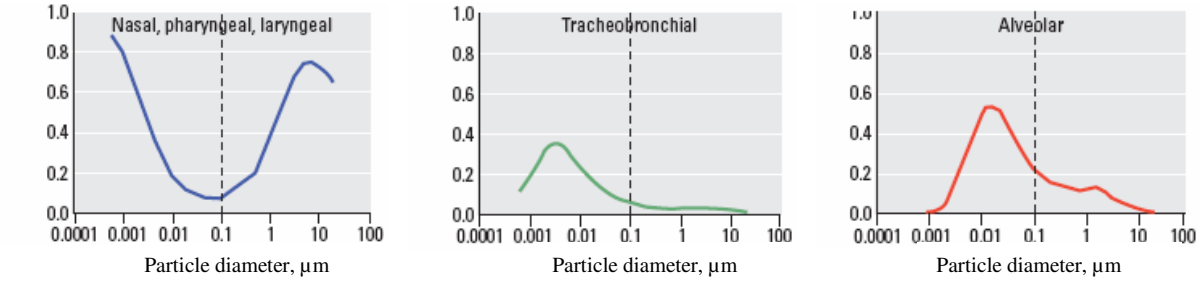

Figure 2. Particle deposition. Regional deposition of particles in the human respiratory tract. (modified from Oberdörster *et al*, 2005, 11)

1.1.4 Clearance & translocation

After deposition the body tries to reduce the retention of inhaled particles by clearing them from the lung. The term “clearance” was introduced to describe the translocation, transformation and removal of deposited particles from the various regions of the respiratory tract. In the upper respiratory tract mainly particles are deposited with an aerodynamic diameter of < 10 µm. In this part of the lung the clearance is performed by coughing. Particles, between 2.5-10 µm, deposited onto ciliated surfaces are removed by the “mucociliary escalator” (12). The particles are transported towards the throat where they are swallowed, thus the gastrointestinal tract can be a secondary target organ of particles after inhalation. The retention time in the bronchial airways is depending on the size of the particles. Kreyling and co-workers showed that the retaining time of particles increases with decreasing diameter (13). The fine and ultrafine fraction of PM mainly deposit in the alveolar region. Alveolar epithelial cells and resident macrophages are the initial target cells for interaction with the particles and are most likely affected by the toxic effects. After deposition, the particles are phagocytosed by macrophages and transported to upper lung regions for mucociliary clearance or the particle-loaded phagocytes enter the lymphatic system (1). Depending on the inhaled concentrations of particles, a considerable part of the particles are taken up by alveolar epithelial cells, which act beside macrophages as initiators of inflammatory responses (14-16).

Ultrafine particles taken up by epithelial cells are considered to escape from the clearance by alveolar macrophages. A fraction of these particles is also able to reach

the systemic circulation (17). Translocation of inhaled ultrafine carbon-particles to the central nervous system was more recently described by Oberdörster *et al* (18).

1.2 Health effects of particles

Adverse health effects by PM were shown initially by epidemiological studies. Concerning the large population size and time needed for performing such studies also animal studies and *in vitro* studies are good complements, especially for investigation of mechanisms and to determine causality.

The lung is the main target organ by PM exposure. Elderly, children and people with existing health problems seem to be more vulnerable (19). Acute health effects caused by PM include increased risk for chronic bronchitis and chronic obstructive pulmonary disease (COPD) as well as worsening of asthmatic symptoms (20, 21). Long-term exposure to combustion-derived PM increases the risk for development of lung cancer. A 10 µg/m^3 increase in $PM_{2.5}$ is associated with 8 % increase of lung cancer mortality (22). Animal studies verified the association between lung cancer and diesel exhaust particles (DEP). Coated polyaromatic hydrocarbons (PAH) are playing a major role herein (23). The “International Agency of Research on Cancer” (IARC) has therefore classified DEP as probably carcinogenic (group 2A).

Additionally, epidemiological studies have found associations between PM exposure and cardiovascular risk factors such as arteriosclerosis, thrombogenesis or decreased heart-rate (24, 25). Peters and co-workers published an increased risk for heart attacks during periods of high air pollution (26).

Also the impact of PM on the human immune system was allocated by epidemiological studies. Increased allergic sensitizations against pollen were observed in areas with increased traffic (27). Pollen, which are bound to particles, are also involved in a higher rate of hospitalizations due to respiratory illness, including asthma (28). Delfino *et al* claimed that particle size and chemical composition are the most important factors for health risk of PM (29).

1.3 Mechanisms of toxicity and protection

In the following part the mechanisms which can lead to toxic effects by particulate matter and the corresponding defence mechanisms for cellular protection are being introduced. The induction of oxidative stress and genotoxicity are playing central roles.

1.3.1 Oxidative stress

The term “oxidative stress” was introduced by Sies, 1991 (30), and describes the disturbance in the oxidant/antioxidant balance, leading to potential damage (figure 3).

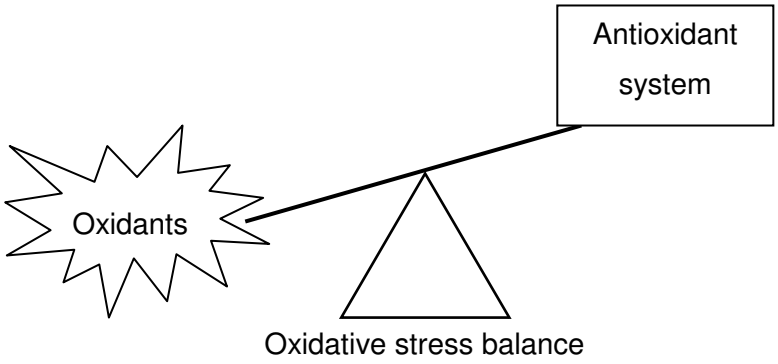

Figure 3. Oxidative stress balance. Oxidative stress is defined as an unbalance in favour of oxidants and disfavouring antioxidants.

Oxidative stress caused by particles is based on an increased availability of reactive oxygen/nitrogen species (ROS/RNS). ROS/RNS are collective terms used to describe oxygen/nitrogen radicals (table 3). Additionally, the term covers non-radicals, which can be easily converted into radicals, such as H_2O_2. Reactive oxygen species can interact with close molecules. This can lead to lipid-, protein- and nucleic acid oxidation. Relating to this, the lifespan of these species is an important issue and range from minutes (hydrogen peroxide) to seconds (peroxide radical) to about a nanosecond, in case of the hydroxyl radical. Oxidative stress is involved in many diseases including inflammation, autoimmune diseases, cancer, diabetes,

neurodegenerative diseases, heart attack and stroke (31, 32). In particle-related toxicity ROS is generated either by the inflammation or by intrinsic particle properties.

ROS		RNS	
Superoxide	$^{\cdot}O_2^-$	Nitric oxide	$^{\cdot}NO$
Hydroxyl radical	$^{\cdot}OH$	Nitrogen dioxide	$^{\cdot}NO_2$
Hydrogen peroxide	H_2O_2	*Nitrite*	NO_2
Singlet oxygen	$^1O_2$	*Peroxynitrite*	$ONOO^-$
Hypochlorous acid	HOCL	Nitrous acid	HNO_2
Ozone	O_3		

Table 3. Overview of ROS and RNS. Non-radicals are in italic.

1.3.2 Antioxidant defence

The extent of damage by ROS/RNS is related to the availability of neutralizing antioxidant defence mechanisms (33). Generally, enzymatic and non-enzymatic mechanisms can be distinguished. Furthermore, antioxidant mechanisms can be divided in extracellular and intracellular defence systems.

After entering the lung, particles first face the extracellular antioxidant mechanisms present in the respiratory tract lining fluid (RTLF, 34). It contains enzymatic antioxidants such as superoxide dismutase (SOD), which catalyses the dismutation of superoxide anion to hydrogen peroxide and O_2. Catalase decomposes H_2O_2 to H_2O. Another enzyme is the gluthatione peroxidase, which reduces hydrogen peroxides by oxidising gluthatione (GSH to GSSG). Also low molecular mass non-enzymatic antioxidants, including ascorbate, uric acid, gluthatione and α-tocopherol are constituent parts of the fluid (35). In the alveolar region of the lung, GSH is considered as a major antioxidant and its synthesis is depending to the activity of gamma-glutamyl-cysteine synthetase (γ-GCS) which is the rate-limiting step.

Metal-chelating proteins such as ferritin or transferrin are also present in the RTLF and are part of the protection against oxidative stress by binding of free transition metals (36). A second line of antioxidant defence consists of intracellular antioxidant systems of both enzymatic and non-enzymatic.

One enzyme which attracts attention for its antioxidant properties is heme oxygenase (HO). It catalyses the degradation of heme, producing carbon monoxide and bilirubin.

Three genes encode three isoenzymes for HO: inducible HO-1 and the constitutively expressed HO-2 and HO-3 (37). HO-1 is highly responsive to oxidative stress and is accepted as a marker for oxidative stress (38). The antioxidant properties are related to the product of the heme degradation, bilirubin, which is considered to be an important antioxidant (39).

If cellular antioxidant defence systems are overwhelmed, one consequence of oxidative stress is the oxidation of DNA.

1.3.3 Oxidative DNA damage

Free oxidants are able to generate a large number of modifications in DNA (40). Generally, the reactions contributing to ROS/RNS-induced DNA damage are oxidation, nitration, depurination, methylation and deamination (41). Currently, more than 100 types of oxidative DNA lesions are known (42). The most prominent lesion is 8-hydroxydeoxyguanosine (8-OHdG), which was discovered by Kasai, 1984 (43). The relatively simple detection of 8-OHdG by HPLC with electrochemical detection (44), made it the most abundantly studied oxidative DNA adduct so far. The presence of 8-OHdG residues can lead to GC to TA transition mutations and therefore may be a part of the initiation of carcinogenesis.

Nowadays, 8-OHdG has been used widely in many studies not only as a biomarker for the measurement of endogenous oxidative DNA damage but also as a risk factor for many diseases including cancer (45). An association between urinary 8-OHdG excretion and lung cancer risk was shown by Loft and co-workers (46).

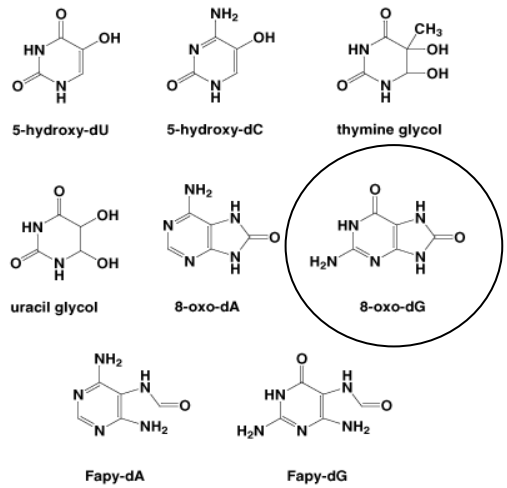

Figure 4. Examples of oxidative DNA base lesions. (from Friedberg E *et al*, DNA Repair and mutagenesis, 2nd edition)

1.3.4 DNA repair

A cell that has accumulated large amount of DNA damage or one with diminished repair activity can enter senescence, apoptosis or unregulated cell division (tumour progression). To maintain genomic integrity before DNA replication, transcription and cell division, the cell is equipped with several DNA repair mechanisms (48). In the following the focus is put on the pathways which are responsible for the repair of oxidative DNA damage.

1.3.5 Base excision repair

The base excision repair pathway (BER) is the main cellular pathway of counteracting the most frequent types of oxidative DNA damage (47). These alterations are highly mutagenic and dangerous for genome integrity. BER is responsible for removal of > 10,000 DNA lesions daily in each human cell (49, 50). The removal of a DNA lesion is initiated by specific DNA glycosylases.

The 8-oxoguanine glycosylase (Ogg1) recognizes specifically the oxidative DNA lesion 8-OHdG; hydrolyze the N-glycosylic bond and is releasing the inappropriate or damaged base. This cleavage generates an apurinic/apyrimidinic (AP) site which is processed by the AP endonuclease 1 (APE/Ref-1) and leads to a single nucleotide

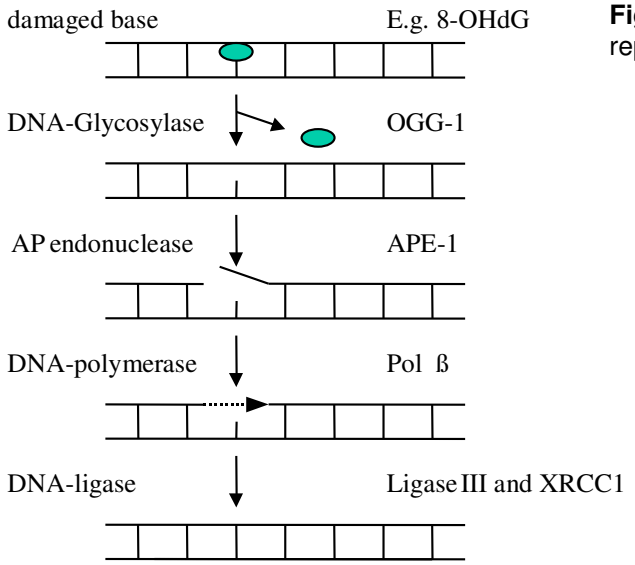

Figure 5. Simplified scheme of the base excision repair pathway.

gap containing a 3´hydroxyl group and 5´phosphate termini. The removal of the phosphate is accomplished by the DNA polymerase ß, which adds the complementary nucleotide to the 3' end of the nick. Finally, the remaining strand nick is sealed by the ligase III. XRCC1 acts as a scaffold protein by bringing the polymerase and ligase together at the site of repair (51, 52).

A second pathway involved in the repair of oxidative DNA damage is the nucleotide excision repair (NER) pathway. NER substrates are bulky DNA adducts, DNA cross-links and some forms of oxidative DNA damage (53). Within the NER process more than 30 proteins are involved. Interestingly, if OGG1 is deficient in the recognition of oxidative DNA lesions, the enzyme CSB, which is related to NER, is acting as a backup system in the recognition of oxidative DNA lesions such as 8-OHdG (54).

One enzyme within BER is of special interest. The APE/Ref-1 is a ubiquitous multifunctional protein and next to its DNA repair function also plays a role in redox regulation (55). It mediates DNA binding of several transcription factors including NFκB (56). APE/Ref-1 is playing an important role in maintaining genomic integrity and in regulating gene expression via redox activation. Transcriptional regulation of this enzyme has been shown to occur via a number of stimuli, including oxidative stress and is therefore involved in the oxidant defence machinery (57, 58). APE/Ref-1 appears to form a link between BER, oxidative stress signalling, transcription-regulation and cell-cycle control (55).

1.4 Particle exposure, inflammation and genotoxicity

Oxidative stress is crucial for the development of adverse health effects caused by PM. One outcome of oxidative stress is DNA damage. Schins and co-workers reviewed the concept of particle-mediated primary and secondary genotoxicity (59, 60).

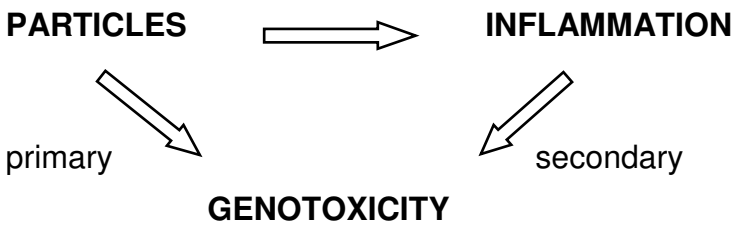

Figure 6. The model of particle-related primary and secondary genotoxicity (modified from 59, 60).

In this model oxidative DNA damage induced by intrinsic generation of ROS is defined as primary, while ROS generation related to particle-elicited inflammation is defined as secondary genotoxicity.
In the following sections the mediators of particle related oxidative stress and their impact on genotoxicity will be discussed.

1.4.1 Redox active transition metals

Particulate matter (PM) is a mixture of solid and liquid anthropogenic and naturally occurring particles of various sizes and composition. For example, combustion-derived ultrafine particles are consisting of a carbonaceous core onto which several substances can be adsorbed. The composition of bonded substances is crucial for the surface reactivity of particles (61). By using electron spin resonance spectroscopy (ESR) the ability of PM to generate hydroxyl radicals was detected, an outcome which was abrogated by the metal-chelator deferoxamine (62, 63). Additionally, several studies showed that iron and other transition metals can lead to the generation of ROS in biological systems (64). A transition metal is defined as an element whose atom has an incomplete d-orbital and is therefore capable of generating ROS via redox cycling with cellular reductants like H_2O_2 (65). The underlying process is called Fenton reaction (36).

$$\cdot O_2^- + H_2O_2 \xrightarrow{Fe} \cdot OH + OH^- + O_2 \quad \text{(net result of the Fenton reaction)}$$

Reduced iron (Fe^{2+}) reduces hydrogen peroxide under the formation of the hydroxyl radical and ferric iron (Fe^{3+}), which can be reduced by reducants such as gluthatione and ascorbic acid back to Fe^{2+}. Beside iron also copper, zinc, vanadium, nickel, titanium and manganese are components of urban air (66).
In addition to the generation of ROS, specific transition metals have been shown to inhibit DNA repair enzymes, like Ogg1 (67), and both contributes to the induction of oxidative DNA damage.

1.4.2 Organic fraction

Primary genotoxicity can be also induced by the organic fraction of PM. Diesel exhaust particles contain a large number of polyaromatic hydrocarbons (PAH) and other organic constituents, which are generated by incomplete combustion processes (68). They can bind to the surface of particles and thereby get access to the body by inhalation. By getting into contact with the biological milieu, PAH are metabolised to redox active quinones by phase I enzymes of the Cytochrome P450 family (69). These molecules are able to generate ROS via redox-cyling (70). Beside the ability of generating ROS, exposure to PAH can also lead to the induction of bulky DNA adducts, after their biotransformation into DNA-reactive metabolites (71).
Several studies confirmed the ability of particle-bound PAH to induce DNA damage. Additionally, the ROS generating properties and related induction of oxidative DNA damage seems to be more important than bulky DNA adduct formation in the genotoxic properties of particle-bonded PAH (72, 73).

1.4.3 Surface area

That size is also an important parameter for the toxicity of inhalable particles was shown by several *in vivo* studies (74, 75). On equal mass basis, ultrafine particles have a much higher total surface area than fine and coarse particles. Thus, the ability of carrying toxic components into the lung is increased. In this regard, comparison between fine and ultrafine particles of same chemical composition revealed that UFP are more potent to cause inflammatory responses (76). Combustion-derived nanoparticles, as carbon black, without notably organic or metals absorbed onto their surface have also been shown to cause inflammation (77). Additionally, Duffin and co-workers showed that surface area is the metric that lead to inflammation by low-toxicity particles (78).
In a cellular free system carbon black nanoparticles were able to generate ROS (79), which assumes that the surface area of particles is sufficient. Anyhow, the underlying chemical basis for ROS generation by low soluble particles is still not fully understood, but catalytic actions with cellular molecules or quantum effects are discussed.

1.4.4 Inflammation

In the model of particle induced genotoxicity, secondary genotoxicity is driven by ROS/RNS generation during particle-elicited inflammation.

Inflammation is a complex biological response to stimuli and lead to the destruction and/or removal of them and to the repair of the damaged tissue. Despite a wide range of stimuli a common pathway for inflammatory responses is existent.

After inhalation of particles, these “invaders” are recognized and cleared by alveolar macrophages (AM). AM are resting cells within the lung and display the major type of phagocytes within the respiratory tract. When AM are overwhelmed in the clearance of e.g. nanoparticles, they are releasing cytokines, which are specific signalling molecules (80). One of these molecules is tumour necrosis factor-α (TNF-α) and plays a pivotal role and completes the first step in the initiation of inflammation (81, 82). The release of TNF-α by AM leads to a further release of chemotatic cytokines as interleukin-8 (IL-8) by phagocytes.

In addition to the ability of AM to render pro-inflammatory cytokines, also alveolar lining epithelial cells are able, after activation by particle characteristics (metals, surface reactivity etc.), to release cytokines (83, 84). Beside the importance for the synthesis of surfactant and the reduction of alveolar surface tension, alveolar type II epithelial cells are contributing to proinflammatory events by releasing chemotatic factors (85, 86). IL-8 acts as a chemoattractant and via the occurring concentration gradient neutrophils are recruited from the respiratory vasculature to the inflamed lung (87). This accumulation of inflammatory cells provides the body to counteract the invasion of xenobiotic compounds.

Particle-induced ROS/RNS generation by phagocytes (AM & neutrophils) was shown for several types of particles (88-90). The generation of ROS is a crucial reaction in phagocytes to degrade internalized pathogens during inflammation. The generation of ROS is called “oxidative burst” (91) and several enzymes contribute to the generation of ROS (92, 93).

The initiation of the respiratory burst during inflammatory response is processed by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (94). It is a membrane-bound five sub-unit complex (figure 7).

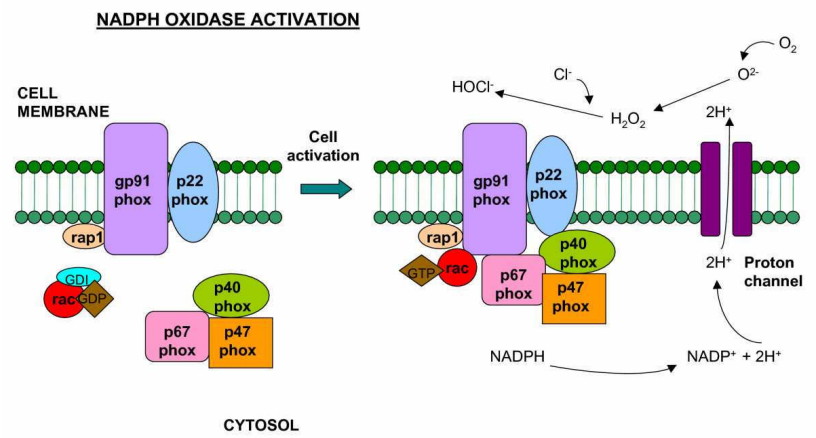

Figure 7. NADPH oxidase. After activation of phagocytes, the cytosolic part binds the membrane-bonded part to form the active complex and ROS are generated.
(from Assari T. 2006. Medical Immunology)

Three of the sub-units ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) are localized in the cytosol, while $p22^{phox}$ and $gp91^{phox}$ are membrane bound. Upon activation, the cytosolic subunits migrate to the membrane parts to form the active complex. The activated NADPH oxidase delivers $^{\cdot}O_2^-$, which can be further converted to H_2O_2 by spontaneously dismutation or by the enzyme superoxide dismutase (SOD) (95). In neutrophils, most of the produced hydrogen peroxide is consumed by the enzyme myeloperoxidase (MPO), which catalyses the reaction of H_2O_2 with chlorine ions to hypochlorus acid. HOCl is highly reactive and are able to oxidise biological molecules (36). A fourth enzyme, the inducible nitric oxide synthase (iNOS), produces nitric oxid ($^{\cdot}NO$). Macrophages, in contrast to neutrophils, produce large amounts $^{\cdot}NO$ upon particle-induced activation. This nitric oxid can react with $^{\cdot}O_2^-$ to form peroxynitrite (96).

This system of enzymatic produced ROS/RNS is a potent machinery for the protection of cellular integrity, but can also contribute to DNA damage in the surrounding epithelial cells if antioxidant and DNA repair capacities are diminished.

1.5 Aims of the study

Epidemiological studies revealed that exposure to particulate matter is associated with an increased risk for adverse health effects (19). 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 COPD and asthma. Oxidative stress and inflammation are also linked to the formation of DNA strand breaks and oxidative DNA damage by inhaled particles. 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.

The main hypothesis of this thesis was that exposure to ambient particles generates ROS, (a) by particle-elicited inflammation or (b) by intrinsic particle properties and that this oxidant generation can lead to oxidative DNA damage and/or to the induction of the oxidative DNA repair pathway. In order to investigate the relationship between exposure, particle characteristics, inflammation and oxidative DNA damage/repair the following *in vivo* as well *in vitro* studies were performed:

Study	**Noxa**	**Question & model**
1)	Ambient PM, collected in Birmingham, UK.	Question: Is the potential to generate ROS and the ability to introduce cytotoxic, inflammatory and oxidative DNA damaging effects more depending either on the size or on the sampling location of ambient PM? Model: *in vitro* : human lung epithelial cells A549
2)	Pure ultrafine carbon particles	Question: Are spark-generated carbon nanoparticles able to induce oxidative DNA damage in mice and rats after short-time inhalation? Model: *in vivo* : female C57Bl/6 mice and male Fischer F344 rats
3)	Quartz particles (DQ12), human carcinogen group I	Question: What is the impact of the NADPH oxidase dependent ROS generation by phagocytic cells on induction of oxidative stress and oxidative DNA damage? Model: *in vivo* : $p47^{phox-/-}$ and wildtype mice, pharyngeal aspiration of DQ12, 24 h *in vitro* : co-culturing of A549 cells and bone-marrow derived neutrophils from wildtype and $p47^{phox-/-}$ mice

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Chapter 2

Oxidant generation and toxicity of size-fractionated ambient particles in human lung epithelial cells

Anton Wessels[1], Wolfram Birmili[2], Catrin Albrecht[1], Bryan Hellack[1], Erich Jermann[1], Gabriele Wick[1], Roy M. Harrison[3], Roel P.F. Schins[1,]

[1]Institut für umweltmedizinische Forschung (IuF) an der Heinrich Heine Universität Düsseldorf gGmbH, Germany; [2]Leibniz Institute for Tropospheric Research, Germany; [3]School of Geography, Earth & Environmental Sciences, University of Birmingham, United Kingdom.

Abstract

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 (3-7, 1.5-3, 0.95-1.5, 0.5-0.95 and < 0.5 µm), collected at four contrasting locations (three urban sites, one remote background) in the UK with a Sierra-Andersen high volume cascade impactor. The H_2O_2-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 LDH assay, and interleukin-8 (IL-8) release as an indicator of their inflammatory potency. Oxidative DNA damage was measured by the formamido-pyrimidine-glycosylase (fpg)-modified comet assay. 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.

2.1 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 physico-chemical 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).

At high concentrations, ROS are known to cause oxidative lesions to genomic DNA, such as the hydroxyl-radical specific and pre-mutagenic adduct 8-hydroxydeoxyguanosine (8-OHdG) (5). Higher rates of 8-OHdG are a well accepted risk factor for the development of cancer. At lower concentrations, ROS have also

been implicated in the ability of PM to activate signalling 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 H_2O_2-dependent formation of •OH is determined by ESR with the aid of the spin-trapping agent 5,5-dimethyl-1-pyrroline-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 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.

2.2 Materials and Methods

Collection of particulate matter by size-fractionated sampling

Size-fractionated samples of ambient PM were 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 (see appendix). For the present investigations we selected a total of 80 PM samples that were collected on the PTFE, i.e. 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 distilled endotoxin-free H_2O 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 filters before and after the extraction procedure, as well as by weighing of the containers after extraction. Freeze-dried samples were stored in the dark at -20°C until furt her use.

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 H_2O_2 (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, USA).

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 foetal calf serum (FCS, Sigma) at 37°C and 5% CO_2. For experiments, cells were seeded into 96-well plates or 24-well plates at a density of 40000 cells/cm^2. After 24 h, cells were synchronized for 16 hours in reduced DMEM (0.1% FCS). PM samples were suspended in complete medium, vortexed for 2 min, sonicated (Sonorex TK52 water-bath; 60 Watt, 35 kHz) for 10 min and directly added to the cells at a concentrations of 100 µg/cm^2 for 4 h 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 analysed by a commercial ELISA Kit (Sanquin, The Netherlands). After 24 h treatment, cell-free supernatants were collected and analysed 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 trypsination 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 analysed as described previously (16)

Statistical analysis

ANOVA with Dunnett post-hoc comparisons were used to test for differences between sampling locations per size fraction (comparing the samples from remote background 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, i.e. *P < 0.05, and **P < 0.01 and ***P < 0.001. ESR values were analysed after log-transformation. Linear regression analysis was used to determine the level of correlation among the different effect parameters. Statistical analyses were performed using SPSS 15.0.

2.3 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.

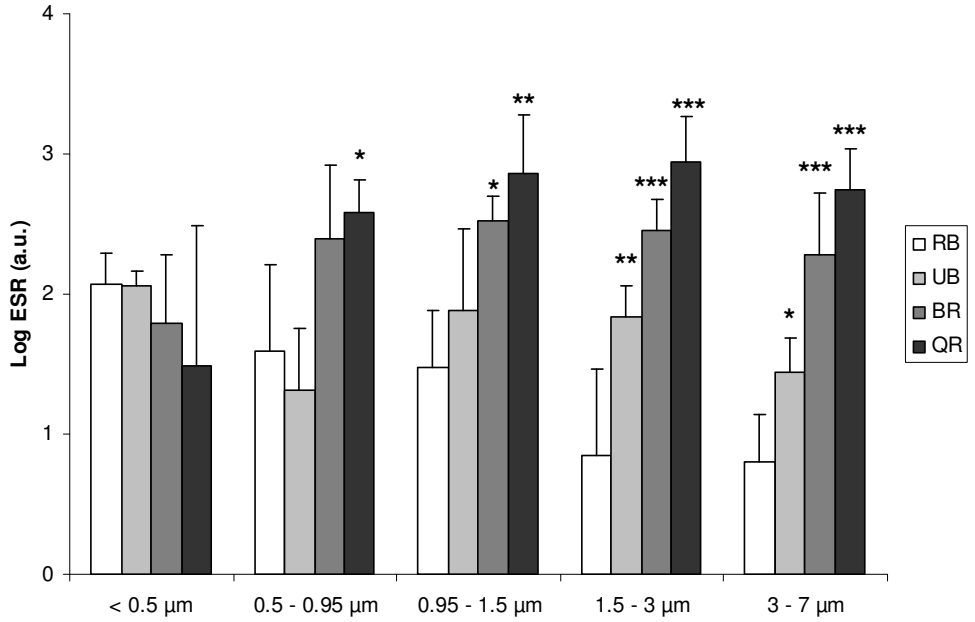

Figure 1. Hydroxyl radical generating properties of the PM samples collected from contrasting locations measured by electron spin resonance (ESR). Results are shown on logarithmic scale. *p<0.05, **p<0.01, ***p<0.001 vs RB of the particular size-fraction.

Marked contrasts were found in the oxidant capacities of samples from both, different particles size fractions and sampling locations. In the coarse particle fractions (3-7 µm, 1.7-3 µm, 0.95-1.5 µm), the oxidant capacity turns out to be a strong function of the observation site, increasing by roughly two orders of magnitude between the remote background and the road tunnel. For the fine particle fraction (< 0.5 µm) the trend is reversed, but below statistical significance. When comparing oxidant capacity across the various size fractions, one can see different trends between different observation sites: At the remote background (RB), oxidant capacity appears to decrease with increasing particle size, while at the roadside (BR) and road tunnel (QR) sites, the reverse is true. Overall, the results show a clear response of the ESR signal towards changes in sampling location and particle size range.

Cytotoxicity of the samples in 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). For the samples from the three urbansites, effects appeared to depend more on sampling location than on size fraction. The highest release of LDH was measured for the Bristol Road and Queensway Road tunnel PM.

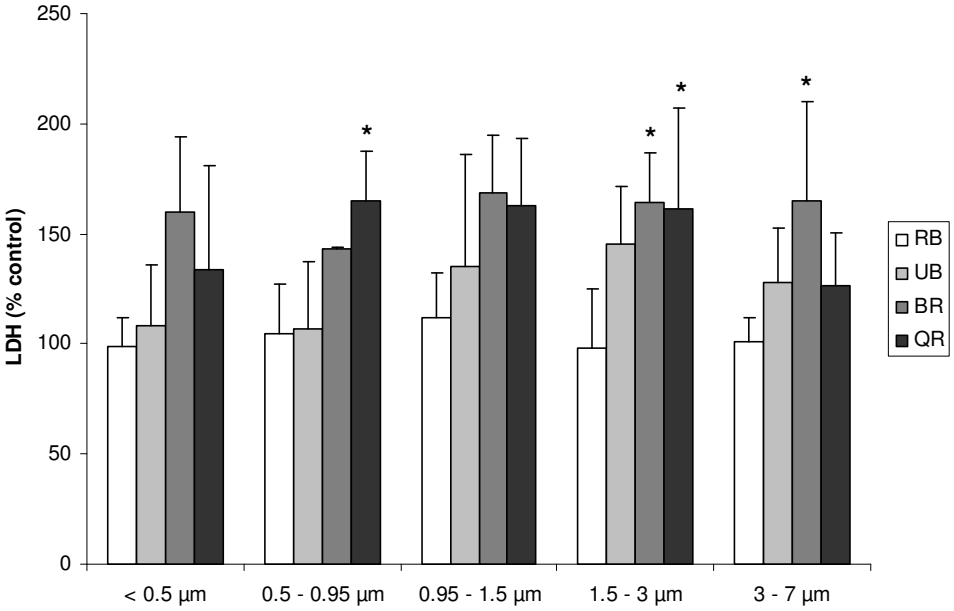

Figure 2. Cytotoxicity measured as release of lactate dehydrogenase (LDH) in A549 cells after treatment with the different PM samples for 24 h at a [C] of 100 µg/cm^2. Data is shown as percentage of untreated control (A549 cells). *p<0.05 vs RB of the particular size-fraction.

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 only few significant differences in IL-8 release were found when comparing specific size fractionated samples from different locations. Irrespective of the sampling location, IL-8 release from the lung epithelial cells tended to be the highest after treatment with the < 0.5 µm particles. The samples collected at the remote background tended to show mild increases in IL-8 release only for the finest fraction (< 0.5 µm). For the samples from the three urban sites, the observed trends in IL-8

release were found to reflect both sampling location and size fraction dependence. The highest cytokine releases were measured for the high-traffic locations, Bristol Road and Queensway Road tunnel.

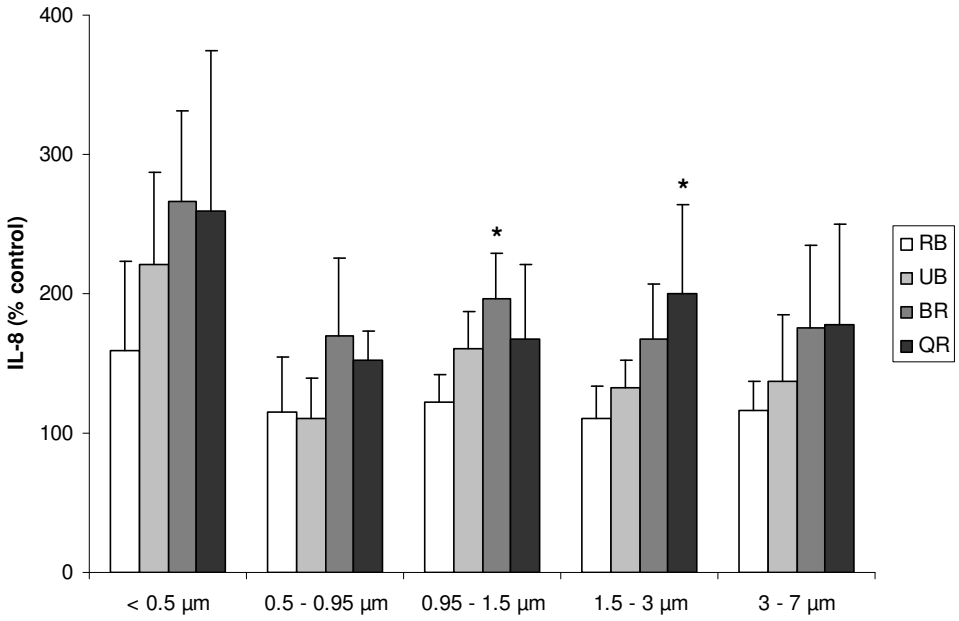

Figure 3. Release of the proinflammatory cytokine interleukin-8 from A549 cells treated for 24h with the PM suspensions with a [C] of 100 µg/cm^2. Data is shown as percentage of untreated control (A549 cells). *p<0.05 vs RB of the particular size-fraction.

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 been 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 & QR). Interestingly, oxidative DNA damage effects appeared to be rather independent of the size fraction of the samples, suggesting that the location is the prime factor to determine the potential of generating oxidative DNA damage to the cells.

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 (see appendix) 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

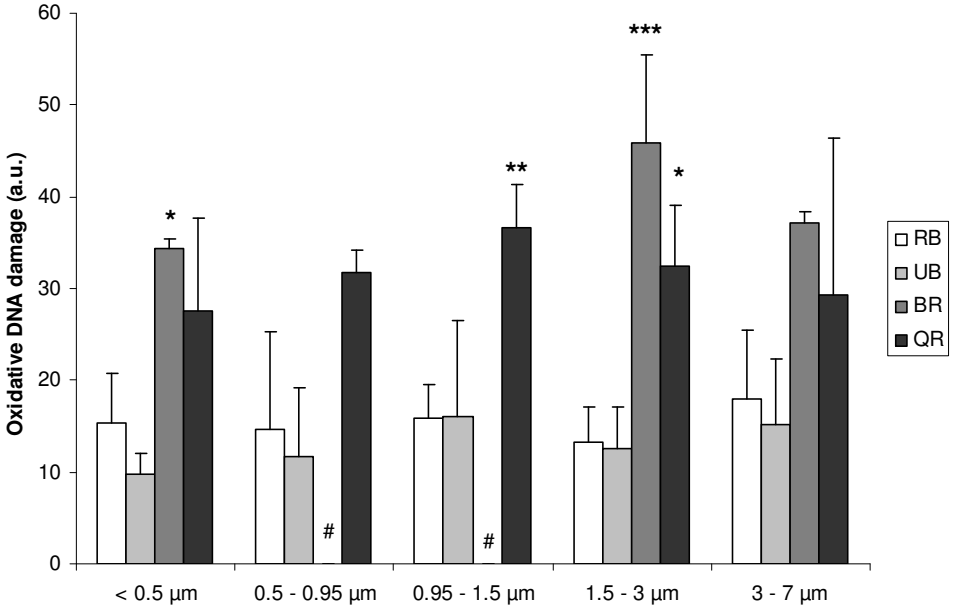

Figure 4. Oxidative DNA damage by the PM samples (4 h treatment) was determined in A549 cells by the fpg-modified comet assay. #not determined. *p<0.05, **p<0.01, ***p<0.001 vs RB of the particular size-fraction.

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, while 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.

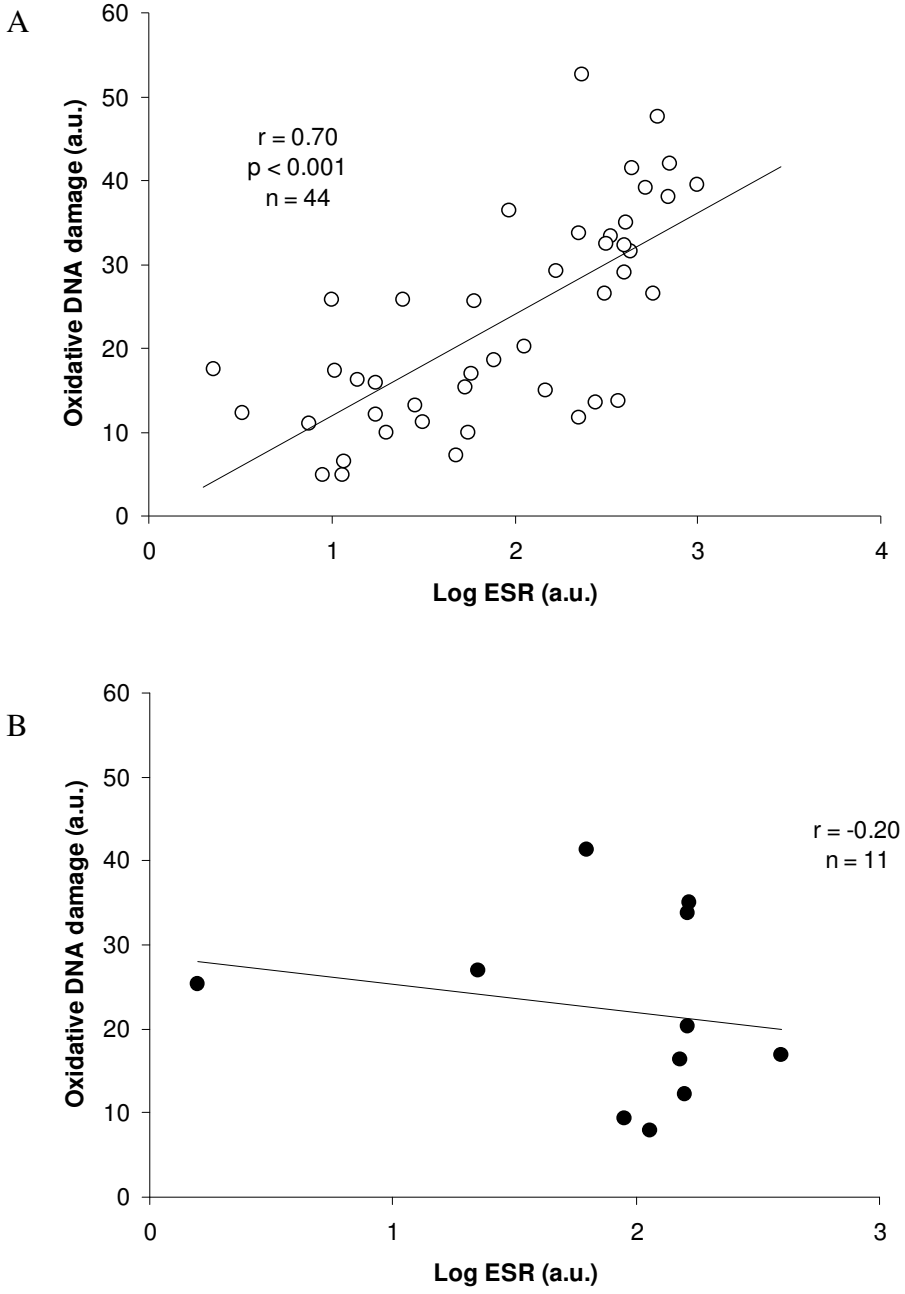

Figure 5. Correlation between hydroxyl radical generating capacity (ESR) and oxidative DNA damage induction. Investigated for all samples except the < 0.5 µm particles (panel A), and for the < 0.5 µm particles only (panel B).

2.4 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 radical generating properties of PM, including Electron spin resonance (ESR) (12, 13). The ESR technique is a method to measure the •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 suggested that the generation of •OH depends rather on the sampling site than on particle size, with the exception of the smallest particle faction (< 0.5 µm 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). However, we did not observe such contrasts for the smallest size fraction (< 0.5 µm). For these particles, we even observed a reversal of the general trend in the ESR signals: The •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 may be 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 •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. Moreover, our ESR method specifically determines the H_2O_2-dependent generation of •OH, for which 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 analysed 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 (15). Interestingly, various metallic constituents are also shown to be able to inhibit DNA repair pathways (20). Taken together, our findings indicate that traffic-derived particles may be particularly harmful to the genomic DNA. However, 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 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 is therefore is a key parameter in localized inflammation. In our study, the particulate size as well the location had an 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 (21, 22). 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) tended to give 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* (21, 23). 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 (24). 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.

In conclusion, 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 endpoints 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.

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Declaration:

The manuscript is submitted to a peer-review journal.

The participation of Anton Wessels for authoring this paper can be estimated in total with 90%.

Table 1.

Characteristics of the sampling campaign and locations (15)

Location	Period	Number of PM samples collected	Characteristics of sampling location
Remote background (RB)	08.08.2002 – 28.08.2002	20	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.
Urban background (UB)	12.07.2002 – 28.07.2002	20	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.
Bristol Road (BR)	08.07.2002 – 17.07.2002	20	Roadside sampling site in Birmingham beside the A38 road, one of the major arteries of radial traffic (22000 vehicles/day).
Queensway road tunnel (QR)	02.07.2002 - 07.07.2002	20	Road tunnel sampling site inside the Queensway underpass, part of the A38 road.

Table 2.

Associations between the oxidant generating capacity of PM samples and toxicity in A549 human lung epithelial cells**.**

Oxidant generating properties were measured by 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 (n.s. = not significant).

	All particle size fractions				***All size fractions except < 0.5***				***< 0.5 µm fraction***			
	ESR	***LDH***	***IL-8***	***DNA***	***ESR***	***LDH***	***IL-8***	***DNA***	***ESR***	***LDH***	***IL-8***	***DNA***
ESR	*	0.366 (n=77) **P<0.001**	0.336 (n=74) **P<0.01**	0.559 (n=55) **P<0.001**	*	0.571 (n=62) **P<0.001**	0.619 (n=60) **P<0.001**	0.701 (n=44) **P<0.001**	*	-0.758 (n=15) **P<0.01**	-0.137 (n=14) n.s	-0.198 (n=11) n.s.
LDH		*	0.094 (n=73) n.s.	0.613 (n=56) **P<0.001**		*	0.251 (n=58) n.s.	0.631 (n=44) **P<0.001**		*	0.062 (n=15) n.s.	0.532 (n=12) n.s.
IL-8			*	0.278 (n=53) **P<0.05**			*	0.642 (n=41) **P<0.001**			*	0.063 (n=12) n.s.

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Chapter 3

Oxidative stress and DNA damage responses in rat and mouse lung to inhaled carbon nanoparticles

Anton Wessels[1], Damien van Berlo[1], Agnes W. Boots[1], Kirsten Gerloff[1], Agnes Scherbart[1], Flemming Cassee[2], Miriam Gerlofs-Nijland[2], Frederik Jan Van Schooten[3], Catrin Albrecht[1], Roel P.F. Schins[1]

[1] Institut für umweltmedizinische Forschung (IuF) an der Heinrich Heine Universität Düsseldorf gGmbH, Germany; [2] National Institute for Public Health and the Environment, Center for Environmental Health Research, Bilthoven, The Netherlands; [3] Department of Health Risk Analysis and Toxicology, Maastricht University, The Netherlands

Abstract

We have investigated whether short-term nose-only inhalation exposure to electric spark discharge-generated carbon nanoparticles (~ 60nm) causes oxidative stress and DNA damage responses in the lungs of rats (152 µg/m^3; 4h) and mice (142 µg/m^3, 4h, or 3 times 4h). In both species, no pulmonary inflammation and toxicity were detected by bronchoalveolar lavage or mRNA expression analyses. Oxidative DNA damage (measured by fpg-comet assay), was also not increased in mouse whole lung tissue or isolated lung epithelial cells from rat. The mRNA expressions of the DNA base excision repair genes OGG1, DNA Pol β and XRCC1 were also not altered. However in the lung epithelial cells isolated from the nanoparticle-exposed rats a small but significant increase in APE-1 mRNA expression was measured. Thus, short-term inhalation of carbon nanoparticles under the applied exposure regimen does not cause oxidative stress and DNA damage in the lungs of healthy mice and rats.

3.1 Introduction

Exposure to ambient particulate matter (PM) has been associated with a broad range of adverse health effects including chronic obstructive pulmonary disease (COPD), cardiovascular diseases and lung cancer (1-3). The fraction of ultrafine particles within the PM mixture has been implicated in the formation of reactive oxygen species (ROS) and oxidative stress, which is considered as an important mechanism in the development and progression of PM-induced diseases (4, 5). In typical urban settings, the ultrafine PM mode is dominated by primary carbonaceous nanoparticles generated from combustion processes, such as diesel exhaust particles (DEP) (6). For the toxicity of such nanoparticles, the surface area is considered as a major determinant. A major factor is the generation of ROS by surface-derived redox reactions which may cause inflammation by activation of signalling pathways resulting in the release of pro-inflammatory cytokines (6, 7). Small sized particles, including those composed of carbon, have been shown to induce inflammation proportional to their surface area (8, 9). Apart from the impact of the surface area also the content of transition metals and organics of PM and combustion-derived nanoparticles has been shown to lead to cellular oxidative stress and pro-inflammatory effects (5, 9-11).

Ultrafine PM, DEP and carbon black nanoparticles - the latter represent engineered nanoparticles that have been applied as a model of ambient nanoparticles (4) - have been shown to enhance the expression of the oxidative stress marker heme oxygenase-1 (HO-1) in cell cultures (5, 12) or in rodent lungs after inhalation (13-15). Analysis of the *in vitro* induction of HO-1 under control of the nrf2 transcription pathway has been proposed as a tool to screen for the oxidative stress potential of engineered nanoparticles in relation to their ROS generating properties (5, 16). A further candidate marker of nanoparticle-induced oxidative stress and inflammation is represented by inducible nitric oxide synthase (iNOS), which is regulated by the redox sensitive transcription factor nuclear factor kappa B (NFκB) (17). Both PM and DEP have been shown to enhance the iNOS mRNA expression in rodent lungs, (18-21). Its increased expression was recently also shown in the mouse peritoneum upon intraperitoneal instillation of silica nanoparticles (22).

Apart from the induction of inflammation, ROS formation is also considered to play a major role in the induction of oxidative DNA damage after inhalation of PM and

nanoparticles. The 8-oxoguanine base lesion is generated in DNA during oxidative stress, and this lesion is pre-mutagenic if not repaired prior to DNA replication (23). Both *in vitro* and *in vivo* studies have shown that PM, DEP as well as various engineered nanoparticles including carbon black can cause oxidative DNA damage (reviewed in 24-28). To cope with DNA damage, cells are equipped with specific DNA repair enzymes. The oxidative DNA lesion 8-OHdG is specifically recognized and repaired by the base excision repair pathway (BER). Hence, Rusyn and colleagues claimed that altered expressions of BER enzymes are sensitive biomarkers for the *in vivo* detection of oxidative stress (29). Major factors involved in BER include 8-OHdG DNA glycosylase 1 (OGG1) (30), apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE-1/Ref-1) (31), DNA polymerase β (DNA Pol β) (32) and X-ray cross complementing group 1 (XRCC-1) (33). Interestingly, Epe and coworkers could recently show that the process of oxidative stress itself can lead to an impairment of oxidative DNA damage repair (34), which may provide a mechanism for the counteracting induction of the mRNA expression levels of specific BER genes.

In vitro and *in vivo* investigations of the DNA damaging effects of nanoparticles have been often performed at high particle concentrations or high exposures, and their potential effects on DNA damage repair in these conditions are poorly addressed. The aim of the present study was to determine whether carbon nanoparticles (CNP) cause oxidative DNA damage responses in the respiratory tract, in view of the oxidative stress paradigm for these materials (16, 35). Therefore we investigated whole lung tissues of mice for markers of oxidative stress and inflammation and for the induction of oxidative DNA damage and DNA BER gene expressions after single (4 hours) or repeated (4 hours on 3 consecutive days) nose-only exposure to CNP generated by electric spark discharge. The same endpoints were also investigated in rats following 4 hours exposure. Additionally, in these animals potential effects were evaluated specifically in the lung epithelial cells that were isolated immediately after inhalation exposure, since these represent critical target cells for the cytotoxic and potential carcinogenic effects of inhaled particles (36).

3.2 Material and Methods

Animals

Specific pathogen (SPF) free female C57BL/6J mice (Taconic, Denmark) and male Fischer F344 rats (Charles River Laboratories GmbH, Sulzfeld, Germany) were used for this study. The animals were housed and maintained in an accredited on-site testing facility under SPF conditions, according to the guidelines of the Society for Laboratory Animals Science (GV-SOLAS). Food and water were available *ad libitum.* At an age of 8 weeks, mice (weight: 20-23 g) and rats (200-250 g) were trained for 1h per day on three consecutive days prior to the experiment to adapt to the inhalation tubes.

CNP generation and exposure characterisation

Rats and mice were exposed by nose-only inhalation to the CNP or HEPA filtered air. The CNP were generated on-site in an argon atmosphere from graphite electrodes in an electric spark discharge generator (Palas Soot Generator, Karlsruhe, Germany). During the inhalation exposures, test atmospheres were continuously monitored using a Condensation Nucleus Counter (3022A, TSI Inc. St Paul MN, USA) as well as a Nanoparticle Surface Area Monitor (NSAM 3550, TSI), and by a Scanning Mobility Particle Sizer (SMPS, TSI) at 30 min measurement intervals. Time-integrated mass concentrations were determined gravimetrically by means of particle collection on two parallel Teflon R2PJ047 filters (Pall corp., Ann Arbor MI, USA), with sampling at a flow rate of 2 l/min. The exposure characteristics in the various inhalation studies are listed in Table 1. The mass, particle and surface area concentration levels showed good agreement for all exposures. A representative size distribution measurement is shown in figure 1.

	Mice, 4 h exposure	**Mice, 3 x 4 h exposure**	**Rats, 4 h exposure**
Mass concentration (µg/m^3)	141	142	152
Number concentration (#/cm^3)	7.7 x 10^6	7.7 x 10^6	7.5 x 10^6
Mean size (nm), (*GSD)	57.5 *(1.86)*	55.3 *(1.80)*	58.8 *(1.65)*

Table 1. Exposure characteristics of the different inhalation studies

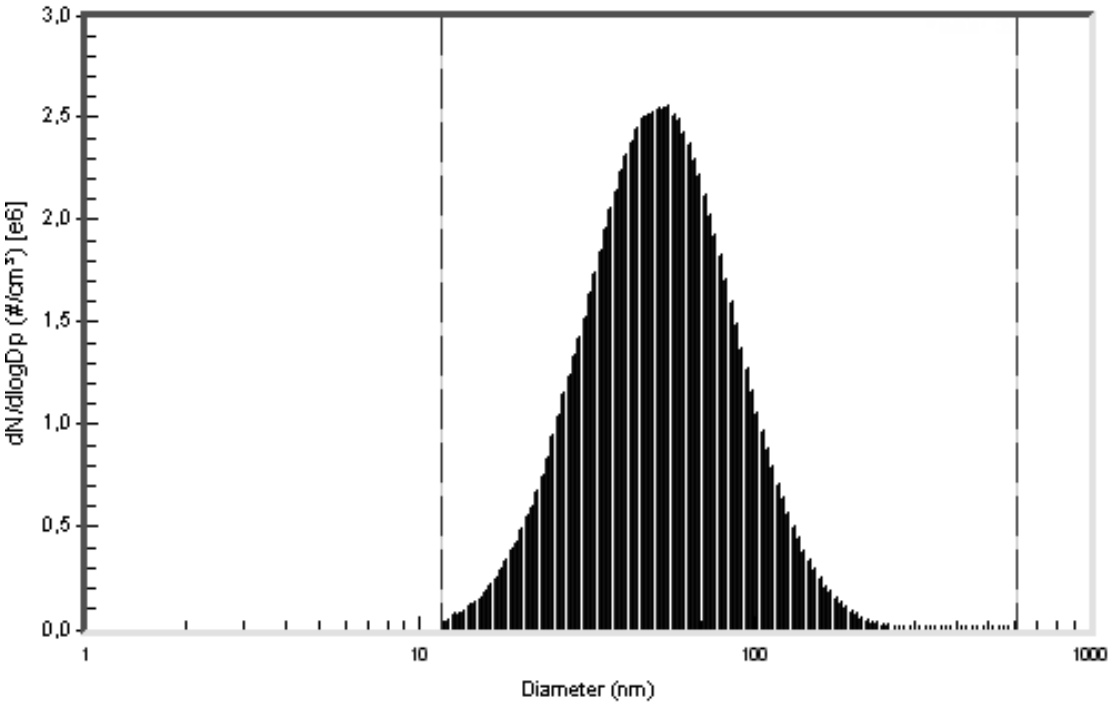

Figure 1. Typical size distribution of the carbon nanoparticles generated by the electric spark discharge generator.

Bronchoalveolar lavage

At the designated time points, mice and rats were deeply anesthetised by a single intraperitoneal injection of Na-pentobarbital, weighed and subsequently exsanguinated via the abdominal aorta. The lungs were cannulated via the trachea and bronchoalveolar lavage was performed *in situ* by infusing the lungs with 5 ml (rat) or 1 ml (mouse) aliquots of PBS. The bronchoalveolar lavage fluid (BALF) was drained passively by gravity when working with rats, while for mice the BALF was reclaimed actively by using a syringe. This procedure was repeated four times and resulted in a BAL volume of 20 ml respectively 4 ml. The BALF was centrifuged twice, i.e. at 300 g to collect cells, followed by 1000 g. The resulting cell-free supernatant was used to assess cytotoxicity by measurement the release of lactate dehydrogenase (LDH) as a marker for cell membrane damage by the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). Total cell number in the BAL was determined after centrifugation by using a hemocytometer chamber (Neubauer) and viability was analysed by trypan blue dye exclusion. BAL-cell differentials were determined by evaluation of cytospin preparations stained with

May-Grünwald/Giemsa (MGG) using light microscopy at 400x magnification. At least 500 cells were counted on each slide.

Isolation of lung epithelial cells from exposed rats

For the determination of oxidative DNA damage and mRNA expression analyses in the rats, their lung epithelial cells were isolated according to the type II lung epithelial cell isolation method developed by Richards *et al.* (37), with some modifications. Following bronchoalveolar lavage, lungs were removed from the rat and pre-washed with 5 ml trypsin (Sigma-Aldrich, Deisenhofen, Germany) via a cannula (37°C, 2.5 mg/ml dissolved in PBS with Ca^{++} and Mg^{++}). The cannulated lung was subsequently attached to a syringe in a retort stand and filled with trypsin solution. The lungs were incubated for 30 min at 37°C, during which the level of trypsin was constantly refilled. After trypsination the lungs were transferred into a sterile petri dish and the lobes of the lung were separated. The digested tissue was minced by the plunger of a syringe and 5 ml FCS (fetal calf serum, Sigma-Aldrich, Germany) was added to arrest the enzyme activity. In all subsequent steps lung preparations and solutions were kept at 4°C. The volume of the lung homogenates was adjusted to 20 ml with PBS and 500 µl DNAse I (4 mg/ml) followed by shaking for 5 min to prevent cell-clotting.

The homogenates were filtered through gauze followed by filtration through nylon filters with a pore size of 150 µm and 30 µm. Further purification was achieved by centrifugation of the final filtrate (adjusted to 20 ml with PBS) onto a discontinuous Percoll gradient (Sigma-Aldrich, Germany) for 20 min at 350 g and 4°C. After centrifugation the layer containing the epithelial cells was carefully removed, washed with PBS, centrifuged at 350 g for 10 min and the pellet was resuspended in 10 ml DMEM medium supplemented with 10% FCS, 1% penicillin/streptomycin solution, 1% L-glutamine (200 mM) and 50 µl DNAse I (4 mg/ml) (Sigma-Aldrich, Deisenhofen, Germany). The cells were plated into 100 mm culture dishes to further enrich the epithelial cell fraction by allowing contaminating fibroblasts, macrophages and neutrophils to attach to the surface. After 1 h incubation at 37°C and 5% CO_2 the supernatant was removed and centrifuged (350 g) to collect non-attached epithelial cells. The cells were resuspended in 3 ml DMEM and counted using a Neubauer chamber, while cell viability was determined by trypan blue dye exclusion.

The percentage of type II epithelial cells present in the isolated fraction was determined by alkaline phosphatase activity staining, as follows. Therefore, cytospin

preparations were dried on air, washed in PBS for 5 min and incubated for 15 min at RT in Fast Red buffer (10 mg Fast Red in 10 ml phosphatase buffer [10 mg naphtol, dissolved in 40 µl DMSO, added to 0.125 M 2-amino-2-methyl-1-propanol, pH 9-10]). Slides were washed again with PBS for 5 min and mounted with Kaisers glycerol gelatine (Merck, Darmstadt, Germany), and type II epithelial cells were counted using light microscopy. As shown in Table 2, this procedure yielded an average purity of type II pneumocytes of ~ 50 %.

	Total cells x 10^5	% Type II cells (Alkaline Phosphatase staining)
Air	30.95 (± 6.46)	52.27 (± 3.59)
CNP	20.56 (± 9.05)	48.55 (± 6.74)

Table 2. Yield and purity of epithelial cell isolation procedure in rats

Preparation of cell nuclei from mouse lungs

Immediately after sacrificing, lungs were removed from non-lavaged mice. The samples were processed for the *in vivo* fpg-modified comet assay based on the method described by Risom *et al.* (13) with the following modifications: Per animal, a quarter of each of five lung lobes was dissected, pooled and transferred to a petri dish filled with 3 ml ice-cold *in vivo* comet assay buffer (IVCAB, containing 0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 10 mM EDTA, pH 7.4), and minced with a plunger of a syringe. The homogenate was pressed through a 40-µm-sieve into a 50 ml Falcon tube, which was subsequently centrifuged at 400 g and 4℃. Supernatant was discarded, and the pellet was resuspended in 200 µl IVCAB.

Measurement of oxidative DNA damage by fpg-modified comet assay in whole lungs of mice and lung epithelial cells isolated form exposed rats

Oxidative DNA damage analysis in cell preparations was assessed immediately after cell-isolation by the fpg-modified comet assay according to the method as described by Speit *et al*. (38). The used protocol for both rodent cell preparations only differed in the first step of the procedure. From the mouse lung extracts, isolated as described above, 25 µl cell suspension was mixed with 590 µl 0.75% low melting point agarose (LMP) dissolved in PBS. Then, 125 µl of this mixture was added onto

1.5% agarose pre-coated slides. For the rat, after epithelial cell isolation as described above, the cell number was determined, as listed in Table 2. The cell suspensions were centrifuged for 5 min at 400 g and resuspended in DMEM at a concentration of 1.5 x 10^6 cells/ml. From this cell suspension, 10 µl cell was mixed with 120 µl 0.5% LMP agarose, and this mixture was then added onto 1.5% agarose pre-coated slides. All subsequent steps of the fpg-comet assay protocol were identical for rat and mouse cells: The slides were covered with coverslips and stored at 4^0C for 10 min to allow solidification. After removing the coverslips, the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10; 10% DMSO and 1% Triton X-100 were added just before use) and stored overnight at 4^0C. On the next day, the slides were rinsed three times for 5 min in fpg-enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8). The slides were then covered with 100 µl of either buffer or fpg-enzyme containing buffer, sealed with a coverslip and incubated for 30 min at 37^0C. Thereafter the coverslips were removed, and the slides were placed in an electrophoresis tank filled with ice-cold electrophoresis buffer (300 mM NaOH, 1mM EDTA, pH >13) for 20 min. Electrophoresis was conducted at 270 mA and 26 V for 10 min. Slides were neutralised 3 x 5 min using neutralisation buffer (0.4 M Tris, pH 7.5). All steps after cell lysis were performed in the dark or under dimmed light to prevent additional DNA damage. After drying for 10 min at RT the slides were stained with 40 µl ethidium bromide (20 µg/ml) and covered. Comet appearances were analysed using an Olympus BX60 fluorescence microscope at 200 x magnification. A comet image analysis software program (Comet Assay II, Perceptive Instruments, Haverhill, UK) was used for quantification of DNA damage by analysis of tail intensities from 50 randomly selected cells. For the evaluation of oxidative DNA damage in the rats, six lungs from each exposure group were evaluated. For the mouse studies, lungs from eight control mice and six lungs from the treated mice were analysed at the 4 h time point. After 3 x 4 h inhalation, lungs from eight mice from each exposure group were analysed.

Measurement of mRNA expression by qRT-PCR

Total RNA was purified from mice lung tissue (i.e. pooled aliquots of the five different lobes of each animal), as well as from the isolated lung epithelial cells from the exposed rats using the Qiagen RNeasy Mini Kit, including RNase-free DNase treatment, as recommended by the manufacturer (Qiagen, Hilden, Germany). From each tissue, 500 ng RNA was used for cDNA synthesis in a reaction volume of 20 µl using the iScript cDNA synthesis kit (BioRad, Munich, Germany). For the PCR reaction the following protocol was used: 5 min 25°C, 30 min 42^0C and 5 min 85^0C. Quantitative RT-PCR was performed using iQ SYBR Green Supermix (BioRad, Munich, Germany) for quantification of the mRNA expression levels. Mouse and rat primers were purchased from MWG Operon, Ebersberg, Germany. The following genes were evaluated in both species: HO-1, iNOS, OGG1, APE-1, XRCC-1, and DNA POL ß. HPRT-1 was used as housekeeping gene. The sequences of all rat and mouse primers used in this study are listed in Table 3. For the PCR reaction, 5 µl of a 1:15 dilution of a cDNA preparation was mixed with 20 µl mastermix (forward/reverse primer, water and SYBR Green Supermix). The probes were placed into the MyiQ cycler (BioRad, Germany), and the PCR reaction was started as followed: 95^0C for 3 min, 40 cycles of 95^0C for 15 s, 60°C for 45 s. We determined relative gene expression by the comparative cycle threshold ($\Delta\Delta C_t$) method, with each gene normalized to HPRT-1 expression (39). Results are reported as fold change over control ± standard error of the mean [($2^{-\Delta\Delta C}{}_t$) ± SEM]. For the mRNA expression analysis in the mouse study, tissues from five animals per exposure group were analysed. For the rat study, we analysed the lung epithelial cells isolated from six animals per exposure group.

Primer	Sequences (5' - 3'), Rat	Sequences (5' - 3'), Mouse
OGG1		
Forward	AAA GTG TGG CTC AGA AAT TCC AA	CCA AGG TGT GAG ACT GCT GAG A
Reverse	AAG AAC AGA TGA AAG AGA AAA GGC ATT	AGC AAT GTT GTT GTT GGA GGA A
APE-1		
Forward	GAA TGT GGA TGG GCT TCG A	TCA AGA AGG CCG GGT GAT T
Reverse	ACA AGA TGT CTG GTG CTT CTT CCT	TTG GGA ACA TAG GCT GTT ACC A
XRCC-1		
Forward	GCC AGC CAT ACA GCA AGG A	ATC TGT GCC TTT GCC AAC ACT
Reverse	TGT CTG GAG GGC TGT GAA ACT	GCA CCC ACT CTT TAC GCA CAA
DNA Polß		
Forward	GCT CGT GGA ACT CGC AAA CT	AAA CAA AGT TCA TGG GTG TTT GC
Reverse	TGC TGC TTT TCT GTA TGC ATT GTA	CGA TTC TCC TGT GTG GAT ATT CC
HO-1		
Forward	GGG AAG GCC TGG CTT TTT T	CCT CAC TGG CAG GAA ATC ATC
Reverse	CAC GAT AGA GCT GTT TGA ACT TGG T	CCT CGT GGA GAC GCT TTA CAT A
iNOS		
Forward	AGG AGA GAG ATC CGG TTC ACA GT	AAC ATC AGG TCG GCC ATC A
Reverse	ACC TTC CGC ATT AGC ACA GAA	CGT ACC GGA TGA GCT GTG AA
HPRT		
Forward	GCC CTT GAC TAT AAT GAG CAC TTC A	AAG ACT TGC TCG AGA TGT CAT GAA
Reverse	TCT TTT AGG CTT TGT ACT TGG CTT TT	AAA GAA CTT ATA GCC CCC CTT GA

Table 3. List of rat and mouse primers used in this study.

Statistical analyses

Data shown in the table represent mean and standard error of the mean (SEM). Exposure associated differences were evaluated by Mann-Whitney U-test, with the levels of significance indicated by asterisks, i.e. *$p < 0.05$ and **$p < 0.01$. Statistical analyses were performed using SPSS 15.0 for Windows.

3.3 Results

BALF analysis

Pulmonary inflammation and cytotoxicity were assessed by bronchoalveolar lavage analysis of mouse and rat lungs after exposure to CNP or air. Results are shown in table 4. For both species, the inhalation of CNP did not result in a significant increase in total BALF cell counts. In contrast, the control animals even tended to have slightly higher total cell numbers than the CNP exposed animals, although these differences did not reach statistical significance. The absence of inflammation was confirmed by differential cell analysis: The specific influx of neutrophils, typical for a particle-induced acute pulmonary inflammatory response, was absent in both mouse and rats after CNP exposure (Table 4). To evaluate pulmonary cytotoxicity after CNP inhalation, the activity of LDH within BALF was measured. Only a very mild, statistically non-significant effect was observed after nanoparticle exposure in both species (Table 4). Thus, it can be concluded that under the inhalation exposure conditions used in our present study, CNP did not cause pulmonary inflammation or toxicity.

	Mouse study		**Rat study**
	1 x 4h	3 x 4h	1 x 4h
Total cells (x10^5)			
Air	0.983 (± 0.149)	0.857 (± 0.175)	24.33 (± 1.15)
CNP	0.792 (± 0.101)	0.720 (± 0.193)	23.67 (± 1.98)
Neutrophils (%)			
Air	0.00	0.03 (± 0.03)	0.00
CNP	0.00	0.00	0.00
Macrophages (%)			
Air	97.29 (± 1.02)	93.38 (± 3.75)	98.67 (± 0.42)
CNP	95.47 (± 2.65)	92.91 (± 3.19)	99.13 (± 0.42)
LDH (% of Air)	102.96 (± 19.56)	102.66 (± 23.80)	102.15 (± 1.53)

Table 4. Total cell number, cell differentials and LDH in BAL of mice and rats after inhalation of CNP.

Analysis of in vivo oxidative DNA damage

The fpg-modified comet assay was used to determine oxidative DNA damage by CNP inhalation in whole lung tissue from the mouse, and specifically within the lung epithelial cells isolated from rat lungs. For the mouse, results after the single 4 h exposure and the three times repeated exposures to CNP are presented in Figure 2. Clear differences in comet tail intensity could be determined in the absence *vs.* presence of the fpg-enzyme, which demonstrated that oxidative DNA damage could be specifically determined in the mouse lung tissues. However, as can be seen in the figure, no significant differences in the DNA damage (i.e. strand breaks and oxidative DNA adducts) were measured between air and CNP treated mice at both designated time points.

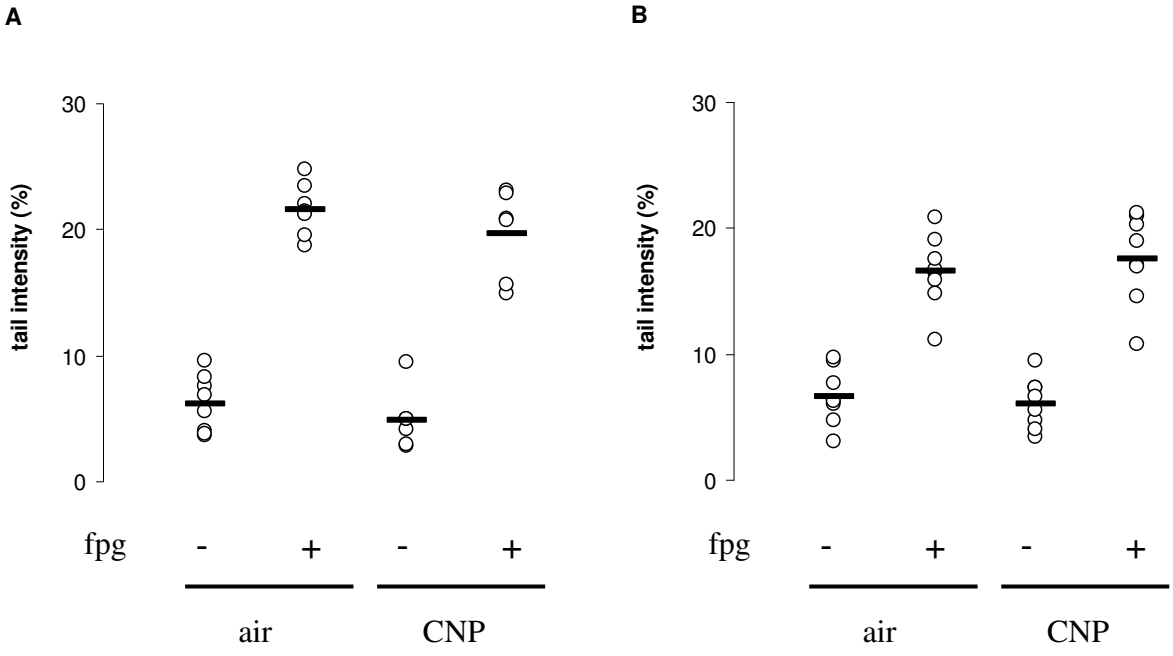

Figure 2. DNA strand breaks and oxidative DNA damage in mouse lung tissue homogenates.
DNA damage was measured by the *in vivo* fpg-modified comet assay after inhalation to carbon nanoparticles (CNP) or air for 4 h (panel A) or for 4 h on three consecutive days (panel B). Each point in the graphs indicates the mean tail intensity value for an individual animal, either in the absence (-) or in the presence (+) of the fpg-enzyme, showing global DNA strand breakage and specific oxidative DNA damage, respectively. The horizontal lines indicate the mean values per exposure group.

In figure 3 the analysis of DNA damage in the isolated epithelial cells from nanoparticles or air exposed rats are shown. In concordance with the observations in the mice, also in the rats no significant increase in DNA strand breakage formation or specific oxidative DNA damage induction was detected in response to CNP inhalation. Interestingly however, the CNP exposure was associated with a small, but statistically significant reduction of DNA strand breakage in the isolated lung epithelial cells from the rats. Taken together, our findings show that short-term inhalation exposure to CNP at the applied particle concentrations, neither in mice nor in rats, led to an induction of oxidative DNA lesions.

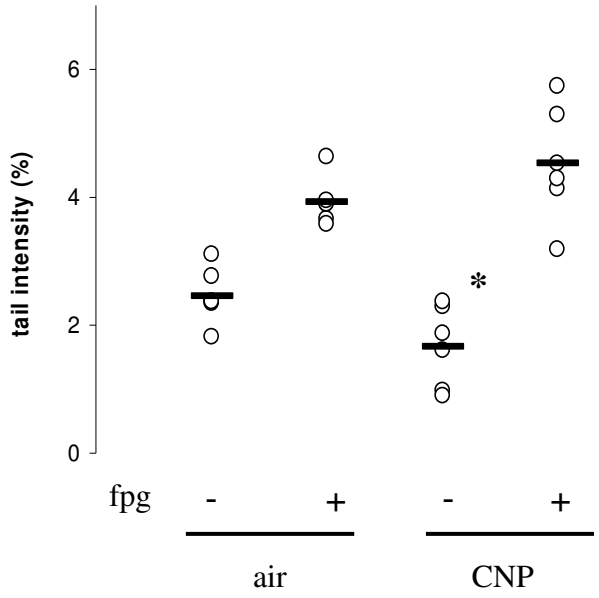

Figure 3. DNA strand breaks and oxidative DNA damage in lung epithelial cells isolated from CNP exposed rats**.**
DNA damage was measured by the *in vivo* fpg-modified comet assay after 4 hours inhalation. Each point in the graph indicates the mean tail intensity value for an individual animal, either in the absence (-) or in the presence (+) of the fpg-enzyme, showing global DNA strand breakage and specific oxidative DNA damage, respectively. The horizontal lines indicate the mean values per exposure group. *$p<0.05$ versus the air exposed animals (Mann Whitney test).

Quantitative RT-PCR

To further evaluate the potential effects of the short-term CNP exposures on pulmonary oxidative stress, inflammation and oxidative DNA damage responses, we evaluated the mRNA expression of a set of marker genes by qRT-PCR. In both species, the mRNA expression of HO-1 and iNOS were determined as markers of oxidative stress. Oxidative DNA damage responses were evaluated in the mice as

well as the rats by analysis of the DNA base excision repair genes OGG1, APE-1, XRCC-1 and DNA POL ß. The results of the whole lung mRNA expressions from mouse lungs after single or repeated exposure are presented in Table 5.

	mRNA fold-change (4 h exposure)	**mRNA fold-change (4 h exposure, 3 days)**
Oxidative stress		
HO-1	1.11 (± 0.07)	1.02 (± 0.07)
iNOS	1.13 (± 0.14)	1.57 (± 0.28)
DNA base excision repair		
Ogg1	1.34 (± 0.07)	1.26 (± 0.10)
Ape-1	1.07 (± 0.02)	1.57 (± 0.11)
Xrcc-1	0.91 (± 0.08)	1.24 (± 0.09)
DNA Pol ß	1.06 (± 0.08)	0.91 (± 0.04)

Table 5. mRNA expression of selected genes in mouse lungs after single or repeated short-term exposures to CNP or filtered air.
Results are reported as HPRT-corrected fold change over control animals' ± standard errors of the mean [($2^{-\Delta\Delta CT}$) ± SEM]. Data represent n = 5 animals per exposure group.

As can be seen in the Table 5, for none of the evaluated genes a significant change in mRNA expression could be detected. Interestingly, however for three out of the four measured BER genes, i.e. OGG1, APE-1, XRCC-1, as well as iNOS, all tended to show increased trends in mRNA expression after repeated CNP exposure in the mouse lungs. In Table 6 the results of the mRNA expression analysis of the lung epithelial cell isolates from the rats after exposure to CNP for 4h are shown. With the exception of DNA Pol ß, the mRNA expression for all genes tended to show mild CNP exposure associated increases. The effect was statistically significant for the DNA endonuclease APE-1. For the other genes effects did not reach statistical significance. Interestingly however, the overall trends in the expression profiles of rats and mice tended to be quite similar.

	mRNA expression fold-change
Oxidative stress	
HO-1	1.18 (±0.18)
iNOS	1.44 (±0.34)
DNA base excision repair	
Ogg1	1.17 (±0.17)
Ape-1	1.46 (±0.09)**
Xrcc-1	1.34 (±0.17)
DNA Pol ß	0.99 (±0.11)

Table 6. mRNA expression of selected genes in epithelial cell isolates from rats exposed to CNP or filtered air**.**
Results are reported as HPRT-adjusted fold change over control animals' ± standard error of mean [($2^{-\Delta\Delta CT}$) ± SEM]. **p<0.01, *p<0.05 (Mann-Whitney). Data represent n = 6 animals per exposure group.

In the mouse experiments (4 h and 3 x 4 h), but not in the rat, the mRNA expression of further genes was measured in air versus CNP-exposed animals, Tumour Necrosis Factor-α (TNFα), Cyclooxygenase-2 (COX2), and Intercellular Adhesion Molecule-1 (ICAM-1) (representing markers of inflammation) and glutathione-S-transferase-µ1 (GSTµ1), γ-Glutamyl-Cysteine-Synthetase (γGST) and NAD(P)H: Quinone Oxidoreductase-1 (NQO-1) (representing antioxidant response genes to oxidative stress). For none of these genes significant expression changes were measured in mouse lungs after single or repeated CNP exposures (data not shown).

3.4 Discussion

In this study we evaluated the effects of short term inhalation exposures to CNP in the lungs of two different species, i.e. the mouse and the rat. As an exposure system we used electric spark discharge, allowing for a controlled repeated dosimetry of nanoparticles into the lungs of the animals by nose-only inhalation. The major outcome of our investigations was an absence of oxidative stress, inflammation, and of the specific induction of oxidative DNA damage in the lung tissue of both rodent species.

Neither in the mice nor in the rats a recruitment of neutrophils, characteristic for an acute inflammatory response to inhaled nanoparticles above threshold concentrations (7), could be observed. The absence of pulmonary toxicity was evidenced by LDH measurements. Effects of short-term inhalation to spark-discharge generated nanoparticles have previously been investigated in mice after 4 h or 24 h whole-body exposure (40). Similar to our current investigations in mice and rats, in this study bronchoalveolar lavage analysis also revealed no inflammation or toxicity in mouse lungs after 4 h exposure to ~50 nm CNP at a concentration of 380 µg/m^3. However, a small increase in neutrophil number and lavage protein levels was observed after 24 h exposure. Using DNA microarray profiling the investigators also detected mild up-regulation of specific genes at either time point investigated, on the basis of which a biphasic, macrophage activation-mediated response to the CNP was proposed (40). In our study however, an inflammatory response was also not observed in the mice that were exposed to CNP on three consecutive days. Moreover, neither in the mice for both time points investigated, nor in the rat inhalation experiment, we could observe changes in the mRNA expression of the sensitive oxidative stress marker HO-1 or of the nitric oxide generating enzyme iNOS. Also the mRNA levels of further marker genes of oxidative stress (i.e. GSTµ1, γGST, NQO-1) and inflammation (i.e. TNFα, COX2, ICAM-1), which we measured in mice only, were found not to be affected by single or repeated CNP exposures (data not shown). Apart from a nearly 3-fold higher exposure concentration and a somewhat lower mean particle diameter, the contrasts between their and our findings may also be explained by the different type of exposure (whole-body *vs.* nose-only) as well as by differences in the mouse strain and their age when investigated (BALB/cJ *vs.* C57BL/6J and 10-12 *vs.* 8 weeks). Taken together, our study showed

that with the exposure regimens used in our study, CNP did not cause significant oxidative stress and inflammation in the lungs of mice or rats.

The major focus of our investigations was to determine whether the short-term inhalation exposures to CNP would result in genotoxic effects in the rodent lungs, represented as increased DNA strand breakage and oxidative DNA damage. Inhalation and instillation studies at high mass exposure concentrations or high dose rate, respectively, have demonstrated that DEP and carbon black nanoparticles induce oxidative DNA lesions and mutagenesis in the lungs of rodents (36, 41-44). Importantly however, these effects are considered to be driven by effects of persistent inflammation, leading to the excessive generation of ROS and nitric oxide by recruited and activated neutrophils and macrophages (reviewed in 24). Moreover, although PM, DEP and carbon black have also been investigated for oxidative DNA damage induction *in vitro*, i.e. in the absence of inflammatory phagocytes (45-48), also here effects are typically found at unrealistically high treatment concentrations required to exceed threshold levels of response (49).

The *in vitro* oxidative damaging effects of nanoparticles have been proposed to relate to their high surface area, causing increased ROS production. Moreover, even in the absence of intrinsic ROS generating properties, nanoparticles may trigger oxidative stress in their target cells via activation of NAD(P)H-like enzyme systems, by disturbance of the electron transport chain of the mitochondria or by calcium-mediated activation of iNOS resulting in nitric oxide generation (5, 26, 50). The generation of ROS and RNS has been clearly implicated in the oxidative attack of DNA (51). Among the currently known oxidative DNA lesions, 8-OHdG is the most prominent and best investigated (52).

To investigate specifically the ability of CNP to induce oxidative DNA damage we used an *in vivo* application of the fpg-modified comet assay, considered as a highly sensitive method to detect such lesions (13). Our investigations revealed that a single or three times repeated exposure to CNP for 4 h was not associated with an observable increase in oxidative DNA damage in the whole lung tissue of mice. However, since the lung is represented by about 50 different cell types, the lack of DNA damaging effects in our mouse experiments may be possible explained by a dilution effect. In view of the average size of the CNP applied in current study, the highest deposition efficiency will be in the alveolar region (7). Thus, the nanoparticles will primarily interact with the alveolar epithelium and resident macrophages.

Therefore, we also performed a CNP inhalation exposure study in the rat, for which we previously have employed a type II epithelial cell isolation procedure to determine cell type specific DNA damage in particle-challenged lungs (53). The type II pneumocytes are the progenitor cells of the alveolar epithelium and known to be target cells for particle-induced tumourigenesis in the rat (36). In previous studies DNA damaging properties after inhalation to (nano)particles have been investigated in either whole lung tissues or in cells obtained by bronchoalveolar lavage representing inflammatory cells (macrophages, neutrophils) for which observed DNA damage increases may not be representative with regard to carcinogenicity risk assessment (26, 49). The analysis of the isolated rat lung cells in the present study showed that a 4 h exposure to 152 µg/m^3 CNP does not lead to increased DNA damage or induction of oxidative DNA damage in carcinogenicity relevant target cells of the lung. In contrast, the CNP exposure was associated with a mildly reduced level of global DNA strand breakage in the isolated epithelial cells.

Apart from the direct investigations of DNA damaging properties of particles also the analysis of DNA repair systems of cells as a feedback mechanism are important to understand the potential influence of nanoparticles on genome integrity. To date, only few *in vivo* studies demonstrated a relationship between particle exposure and repair pathways. The DNA-glycosylase enzyme OGG1 specifically recognizes oxidative DNA lesions and removes them as a first step within the BER pathway. Enhanced expression of OGG1 was described after exposure to high doses of DEP (13, 41). Although this suggests that the induction of OGG1 is an important cellular response to oxidative stress by particles, it should be emphasized that the DEP exposures also caused pulmonary inflammation. In contrast, in our present study Ogg1 mRNA levels were not significantly affected after CNP exposure in both rodent studies suggesting that its expression is not affected in non-inflamed lung tissues. Apart from the DNA-glycosylase we also measured the mRNA expression profiles of BER pathway genes downstream of Ogg1. For Xrcc1 and DNA Pol ß no significant expression changes were detected in either mice or rats after CNP exposure. Interestingly however, while not affected in whole lung tissue of CNP exposed mice, a small but statistically significant increase in the mRNA expression of APE-1 was found in the lung epithelial cells from the CNP exposure rats. APE-1/Ref-1 is a ubiquitous multifunctional protein that possesses both DNA repair activity and redox regulatory activity (54). In addition to the already mentioned role in the base excision repair

pathway, the enzyme is involved in mediating DNA binding of a number of transcription factors including NFκB, AP-1 and p53 in association with oxidative stress (54). Former *in vitro* studies have revealed increased APE-1/Ref-1 expression after treatment of mesothelial cells or macrophages with asbestos (55, 56). In a previous study in our laboratory, intratracheal instillation of highly inflammogenic crystalline silica particles was found to induce APE-1/Ref-1 protein expression in rat lungs, specifically in alveolar macrophages and to a lesser extent in lung epithelial cells (57). In relation to its endonuclease function, it is interesting to note that in our current study, parallel to the increased APE-1/Ref-1 expression, lower background DNA damage was found in the lung epithelial cells from the CNP exposed rats. However, one should be cautious about the direct relevance for the *in vivo* situation since the observed effects may also reflect, at least to some extent, responses to the various isolation steps required to harvest the epithelial cells from the rat lungs. Moreover, in a recent study we could not confirm an induction of APE-1/Ref-1 mRNA expression 24 hours after high-dose quartz instillation in whole mouse lungs (58, submitted). Further studies are needed to determine the relevance of APE-1/Ref-1 mRNA expression analysis as a marker of nanoparticle-induced oxidative stress and/or DNA damage.

In summary, in the present study we could show that short-term inhalation exposures of mice and rats to carbon nanoparticles, in the absence of marked oxidative stress and inflammation, do not result in DNA damage, as measured by the highly sensitive fpg-modified comet assay. Most likely, the applied exposure regimens were insufficient in terms of both particle mass and particle number to exceed a threshold, under which cellular defence systems can prevent such damage. Although the observed increased mRNA expression of APE-1/Ref-1 in rat may be an cell isolation artefact, the tendencies of further BER genes to be up-regulated in both rodent models investigated in our study, might be in support of this threshold concept. Further studies are therefore required to determine the role and impact of DNA repair pathways in nanoparticle-exposed tissues in relation to appropriate exposure conditions. We consider our current findings to be of importance with regard to current risk assessment debate in the nanotoxicology field, specifically as the number of *in vitro* genotoxicity studies with unintentionally generated as well as engineered nanoparticles is steadily rising.

Acknowledgements

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Declaration:

The manuscript is submitted to a peer-review journal.

Most experimental work presented, was done by Anton Wessels. The impact on authoring this paper can be estimated to 80%.

3.5 References

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Chapter 4

Neutrophil-derived oxygen species contribute to oxidative stress and DNA damage induction by respirable quartz particles

Damien van Berlo[1]; Anton Wessels[1]*; Agnes W Boots[1,2]; Verena Wilhelmi[1]; Agnes M. Scherbart[1]; Kirsten Gerloff[1]; Catrin Albrecht[1]; Roel P.F. Schins[1]*

** Equal contributions*

[1]Institut für umweltmedizinische Forschung (IUF) an der Heinrich-Heine-Universität Düsseldorf gGmbH, Germany; [2]Department of Pharmacology and Toxicology, Maastricht University, Netherlands

Abstract

The carcinogenesis of respirable quartz is considered to be driven by reactive oxygen species (ROS) generation in association with chronic inflammation. We investigated the contribution of ROS to acute oxidative stress and DNA damage in the lungs of quartz-exposed p47$^{phox-/-}$ mice, which are characterised by impaired phagocyte-derived superoxide anion generation. C57BL/6J (WT) and p47$^{phox-/-}$ mice were treated with 100mg/kg b.w. DQ12 quartz or saline via pharyngeal aspiration. After 24 hours, pulmonary inflammation, oxidative stress and DNA damage responses were evaluated. Bone marrow-derived neutrophils from WT and p47$^{phox-/-}$ mice were used for parallel *in vitro* DNA damage investigation in coculture with A549 human alveolar epithelial cells. Quartz treatment of cocultures containing WT neutrophils, but not of cocultures incorporating p47$^{phox-/-}$neutrophils, caused increased oxidative DNA damage in the epithelial cells. Quartz also induced acute inflammation characterised by a remarkably similar influx of neutrophils in the lungs of WT and p47$^{phox-/-}$ mice. In contrast, significant increases in mRNA expression of the oxidative stress markers HO-1 and gamma-GCS were only observed in the treated WT animals. Oxidative DNA damage in lung tissue however, was not affected by the quartz exposure and also did not differ between p47$^{phox-/-}$ and WT mice. Similarly, no *in vivo* differences in mRNA expression of the DNA repair genes OGG-1, APE-1, DNA Polβ and XRCC1 were found. Our study demonstrates that neutrophil-derived ROS significantly contribute to pulmonary oxidative stress after acute quartz exposure, although the associated induction of oxidative DNA damage could only be shown *in vitro*.

4.1 Introduction

Chronic exposure to respirable crystalline silica, the most abundant form of which is quartz, occurs in various occupational circumstances such as mining, sandblasting, highway construction and stonemasonry and can lead to a variety of severe pulmonary diseases like silicosis and chronic obstructive pulmonary disease (COPD) (1, 2). Moreover, respirable quartz is classified as human carcinogen by IARC (2). Persistent formation of reactive oxygen species (ROS) and resultant oxidative stress are considered to play a major role in the development and progression of silicosis (3) as well as in the genotoxic and carcinogenic effects of inhaled quartz particles (4, 5). Particle-induced oxidative stress can take place via two routes. Although it can result from inherent properties of the particles themselves, ROS are also produced in the lung in significant amounts during the oxidative burst of inflammatory cells (e.g. macrophages and polymorphonuclear neutrophil granulocytes [PMNs]).

Quartz has been shown to generate ROS in a cell-free environment (6-8), and there is substantial evidence to support the ability of quartz to induce oxidative stress. This is for instance measured as oxidation and depletion of the major pulmonary antioxidant glutathione, or the expression of γ-glutamyl cysteine synthetase (γ-GCS), the rate-limiting enzyme for its synthesis (9, 10). Oxidative stress by quartz is also evidenced by the induction of the sensitive oxidative stress marker heme oxygenase-1 (HO-1) (11-13) and oxidative DNA lesions (8, 13). In addition to manifesting oxidative properties associated with surface chemistry, quartz is also a potent inflammogen. Acute exposure to respirable quartz causes a rapid influx of neutrophils and the upregulation of various proinflammatory mediators, including cytokines and chemokines (14-17).

The inflammogenic potential of quartz is considered to be crucial for the development of subsequent pathology, including fibrosis and lung cancer. The excessive formation of ROS generated from activated phagocytes during inflammation is considered to cause oxidative stress in silicotic lungs (18), and has been implicated in oxidative DNA damage induction and mutagenesis (4, 5, 19). These cells mainly produce ROS via the NADPH oxidase enzyme complex consisting of 7 family members: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2. The isoform predominantly expressed by professional phagocytes, NOX2 or $gp91^{phox}$, especially serves the purpose of host defense (20). To form a biologically active enzyme complex, multiple

additional subunits such as $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ need to be recruited from the cytosol to the membrane-bound subcomplex consisting of NOX2, $p22^{phox}$ and Rac. The essential role of NOX2 in host defense is supported by the notion that people lacking NOX2, $p22^{phox}$, $p47^{phox}$ or $p67^{phox}$, develop chronic granulomatous disease, a condition characterised by a strong occurrence of infections (21). A further deficiency was recently discovered as autosomal recessive mutation of the $p47^{phox}$ subunit (22).

Although *in vivo* studies indicate that the induction of pulmonary oxidative stress and associated DNA damage by quartz results from the oxidative attack by phagocyte-derived ROS, *in vitro* studies also demonstrate that quartz particles are capable to inducing such effects in the absence of inflammatory cells (reviewed in 23). However, the contribution of each of these two pathways, as well as their potential interactions, in driving pulmonary inflammation and the causation of oxidative stress and DNA damage induction upon inhalation of quartz particles is currently still unknown. The aim of the current study was to evaluate the role of NOX2-mediated ROS formation on quartz-induced inflammation, oxidative stress and oxidative DNA damage. To investigate this aim, a mouse model featuring impaired phagocyte ROS generation due to the knockout of the $p47^{phox}$ NOX2 subunit was employed. Wildtype (WT) and $p47^{phox-/-}$ knockout mice were treated with quartz via pharyngeal aspiration and evaluated for lung inflammation, oxidative stress and DNA damage responses. In parallel *in vitro* investigations, DNA damage by quartz was also evaluated in cocultures of A549 human lung epithelial cells and freshly isolated bone marrow neutrophils from either WT or $p47^{phox-/-}$ mice.

4.2 Materials and Methods

Chemicals

Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin, ethidium bromide, fetal calf serum (FCS), penicillin/streptavidin solution, phosphate buffered saline (PBS), Ca^{++}/Mg^{++}-containing Hanks' balanced salt solution ($HBSS^{(+/+)}$), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), H_2O_2, Trypan blue, Percoll, trypsin, lucigenin, agarose, low melting point agarose, phorbol-12-myristate-13-acetate (PMA) and bovine serum albumine (BSA) were provided by Sigma (Deisenhofen, Germany). Sulfamethoxazol/trimethoprim (Cotrim) was provided by Ratiopharm (Ulm, Germany). Trizol was obtained from Invitrogen (Karlsruhe, Germany), while RNeasy® mini kit and RNase-free DNase set were bought from Qiagen, Valencia, CA, USA. The iScript cDNA Synthesis kit and SYBR© Green Supermix were obtained from Biorad, CA, USA. Luminol was ordered from Roth (Karlsruhe). Fpg enzyme was kindly provided by Dr. Andrew Collins, Institute for Nutrition Research, University of Oslo, Norway. All other chemicals were supplied by Merck (Darmstadt, Germany). qRT-PCR primers were designed using Primer Express software and ordered from Operon.

Particles

Dörentruper Quartz, ground product 12 (DQ12), batch 6, with a mean diameter of 0.96 µm, was used as the particle sample. Particles were baked for 24 h at 220 °C to remove possible endotoxin contamination. Before exposing the mice, particles were suspended in saline and sonicated for 10 min (Sonorex TK52 waterbath; 60 W, 35 kHz). In the coculture studies, particles were sonicated in HBSS immediately prior to the treatment.

Animals

The specific pathogen-free (SPF) C57BL/6J wildtype and $p47^{phox-/-}$ transgenic mice used for the study were obtained from Taconic (Lille Skensved, Denmark). Animals were divided in 4 groups: DQ12-treated WT mice, saline-instilled WT mice, DQ12-treated $p47^{phox-/-}$ mice and saline-instilled $p47^{phox-/-}$ mice. The animals were kept and maintained in an accredited on-site testing facility (safety level 1), according to the guidelines provided by the Society for Laboratory Animals Science (GV-SOLAS).

Water containing antibiotics (800 mg sulfamethoxazol, 160 mg trimethoprim/L) and food were available *ad libitum*. WT and p47$^{phox-/-}$ mice were housed under SPF conditions on hardwood or alpha-dri (Lillico, UK) bedding respectively, in plastic cages in an air-conditioned animal room (23 ± 2 ℃) with a regular 12 h light/dark cycle.

Isolation of bone-marrow derived PMN

Mouse bone marrow neutrophils were obtained based on to Boxio et al (24). Both hind legs from the sacrificed animals were removed at the pelvic bone followed by removing the tissue and muscles from the bones. To isolate PMNs the distal tip of the femur and tibia bone was cut off. Sterile PBS supplemented with 1 % penicillin/streptomycin was forced through the bone using a syringe with a 0.4 x 19 mm needle. The obtained cell suspension was washed through a 100 µm nylon mesh cell strainer (BD Falcon) and then centrifuged at 400 g for 5 min (4℃), the pellet was resuspended in 1 ml HBSS-BSA. A three-layer Percoll gradient diluted in PBS, consisting of 78% Percoll, followed by 69% and topped by 52%, was prepared and kept at 4℃. The cell suspension was added on the gradient using a syringe. PMN were harvested from the middle interface after centrifugation (1500 g; 30 min; 4℃). After washing with HBSS-BSA, lysis of the remaining erythrocytes (155 mM NH_4Cl, 10 mM $KHCO_3$, 1.0 mM EDTA, H_2O; pH 7.4) and further washing, PMN were resuspended in HBSS, diluted to the required concentration and finally used for coculture studies and chemiluminescence. The PMN yields were 12.94 (± 5.22) for WT mice and 10.67 (± 4.14) for p47$^{phox-/-}$ mice.

Chemiluminescence

ROS formation by quartz-treated PMNs was analyzed using lucigenin- and luminol-enhanced chemiluminescence (25, 26). Briefly, freshly isolated PMNs were suspended in ice-cold HBSS at 1 x 10^6 cells/ml. From this cell suspension, 50 µl was added to a white maxisorp 96-well plate (Nunc, Germany) at 37ºC containing 50 µl of 20 or 40 µg/cm^2 DQ12. Next, 100 µl of either lucigenin or luminol (both 5 x 10^{-4} M) was added and measurement was immediately started. Chemiluminescence was recorded for 60 min at 37ºC using a Multi-Bioluminat luminometer (Berthold, Germany) and expressed as area under the curve. The experiment was performed three times.

Coculture studies

A549 human lung epithelial cells were seeded in a 24-well plate at 8 x 10^4 cells/ml. Cells were then cultivated at 37°C and 5% CO2 in DMEM containing 10% FCS/1% penicillin/1% streptomycin/1% glutamine for 1 day until they reached approximately 90-95% confluence. Freshly isolated PMN from wildtype or $p47^{phox-/-}$ mice were then added to the A549 monolayers at a cell number ratio of 1:2 (A549 : PMN). These cocultures, as well as A549 cells in monoculture, were immediately treated with DQ12 (100 µg/cm^2) or H_2O_2 (100 µM) in HBSS for 2 h. Following incubation, the cell monolayers were rinsed three times in PBS to remove all neutrophils and/or particles, as verified by microscopy. Oxidative DNA damage was analysed in the A549 cells after their trypsination, centrifugation and resuspension in fresh complete culture medium by the fpg-comet assay as described elsewhere (13).

Pharyngeal aspiration study

Animals (6-10 weeks) were anaesthetised by isoflurane inhalation and treated with 100 mg/kg b.w. DQ12 by pharyngeal aspiration (27). Control animals received the vehicle control, saline. Twenty-four hours post-aspiration, animals were deeply anesthetised with pentobarbital (50 mg/kg b.w.) and sacrificed through exsanguination via the A. abdominalis. To assess inflammation, bronchoalveolar lavage was performed as described below. Following lavage, lungs were removed, and aliquots of five lobes per animal were dissected and pooled for DNA damage and mRNA expression analyses. Macroscopic evaluation showed that the lungs of all included $p47^{phox-/-}$ animals were free of granuloma.

Bronchoalveolar lavage

After cannulation of the lung via the trachea, bronchoalveolar lavage (BAL) was performed by performing four consecutive washes with 1 ml saline. The total cell number in BAL fluid (BALF) obtained from five mice from each group was determined by a haemocytometer. Cytospin preparations were stained with May-Grünwald/Giemsa for cell differential analysis.

In vivo fpg-comet assay

In vivo DNA damage was measured in homogenised lung tissue obtained from 5 quartz-treated and 5 saline-treated animals from both wildtype and p47$^{phox-/-}$ mice. The samples were processed for the fpg-comet assay based on the method described by Risom et al. (28) with the following modifications: per animal, a quarter of each of five lung lobes was dissected and pooled in an Eppendorf cup containing 300 µl *in vivo* comet assay buffer (IVCAB, containing 0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 10 mM EDTA, pH 7.4), on ice. Then, lung tissue was thoroughly homogenised on a Petri dish, in 3 ml ice-cold IVCAB. The homogenate was pressed through a 40 µm sieve into a 50 ml Falcon tube, which was subsequently centrifuged at 400 g and 4°C. Supernatant was discarded and the pellet was resuspended in 200 µl IVCAB. 20 µl cell suspension was added to 590 µl 0.75% low melting point (LMP) agarose, resulting in a concentration of 1.5×10^6 cells per ml. From here on, the comet assay protocol was identical to the *in vitro* protocol described elsewhere (13).

Quantitative RT-PCR

Lung tissue of 5 quartz-treated and 5 saline-treated wildtype and p47$^{phox-/-}$ mice were homogenised in Trizol. For each animal, therefore a pooled sample consisting of a quarter of each of five lung lobes was used. The RNA was extracted as recommended in the manufacturer's instructions. Purification of the obtained RNA was performed using the RNeasy method coupled to treatment with DNase. The purity and amount obtained were determined by spectrophotometry at wave-lengths of 230, 260, 280, and 320 nm. Then, using the iScript cDNA Synthesis kit (BioRad, CA, USA), cDNA was synthesised from 0.5 µg RNA per sample. Before qRT-PCR, cDNA was diluted 15x in RNase-free water. Primers for the oxidative stress markers HO-1 and γ-GCS, the DNA base excision repair (BER) genes APE-1, OGG1, DNA Pol ß, XRCC1 and the housekeeping gene HPRT were designed using Primer Express software (Applied Biosystems). Sequences of the employed primer pairs were the following:

for HO-1:	5'-CCTCACTGGCAGGAAATCATC-3	(forward)
	5'-CCTCGTGGAGACGCTTTACATA-3	(reverse)
for γ-GCS:	5'-CGACCAATGGAGGTGCAGTTA-3	(forward)
	5'-ACCCTAGTGAGCAGTACCACGAA-3	(reverse)

for OGG1:	5'-CCAAGGTGTGAGACTGCTGAGA-3	(forward)
	5'-AGCAATGTTGTTGTTGGAGGAA-3	(reverse)
for APE-1:	5'-TCAAGAAGGCCGGGTGATT-3	(forward)
	5'-TTGGGAACATAGGCTGTTACCA-3	(reverse)
for DNA Polß:	5'-AAACAAAGTTCATGGGTGTTTGC-3	(forward)
	5'-CGATTCTCCTGTGTGGATATTCC-3	(reverse)
for XRCC1:	5'-ATCTGTGCCTTTGCCAACACT-3	(forward)
	5'-GCACCCACTCTTTACGCACAA-3	(reverse)
for HPRT:	5'-AAGACTTGCTCGAGATGTCATGAA-3	(forward)
	5'-AAAGAACTTATAGCCCCCCTTGA-3	(reverse).

Then, 2.5 µl of 0.3 µM forward and reverse primer was mixed with 2.5 µl water and 12.5 µl SYBR© Green Supermix (Biorad) and added to 5 µl sample, yielding a final volume of 25 µl per well of a 96-wells PCR plate. qRT-PCR was performed using a MyiQ Single Color real time PCR detection system (BioRad), according to the following protocol: denaturation 95ºC for 3 min was followed by 40 cycles at 95ºC (15 seconds) and 60ºC (45 seconds). After PCR, a melt curve (60-95ºC) was produced for product identification and purity. PCR efficiency of all seven primer sets, assessed by measuring cDNA dilution curves, was at least 90%. Data were analysed using the MyiQ Software system (BioRad) and were finally expressed as relative gene expression (fold increase compared to control animals) according to the 2-ΔCt method (29).

Immunohistochemistry

Lungs of three additional animals per treatment group and genetic background were instilled *in situ* with 4% paraformaldehyde/PBS (pH 7.4) using a 1 ml syringe, removed, fixed, dehydrated, and paraffin embedded. Lung sections were stained for OGG-1 after rehydration and antigen retrieval using citrate buffer. Binding of the primary anti-OGG-1 antibody (1:1000, Novus Biologicals, Littleton, USA) was detected using a secondary biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories, USA) followed by the streptavidin-biotin-complex according to the manufacturer's protocol (Vector Laboratories, USA). Diaminobenzidine (Sigma, Deisenhofen, Germany) was used as a substrate, and the slides were counter stained with hematoxylin. In order to control for unspecific binding, additional sections

were incubated with rabbit IgG instead of primary antibody at the same IgG concentrations. Microphotographs were prepared by oil immersion at an original magnification of 1:1000 using a light microscope (BX60) and analysis software (Olympus, Hamburg, Germany).

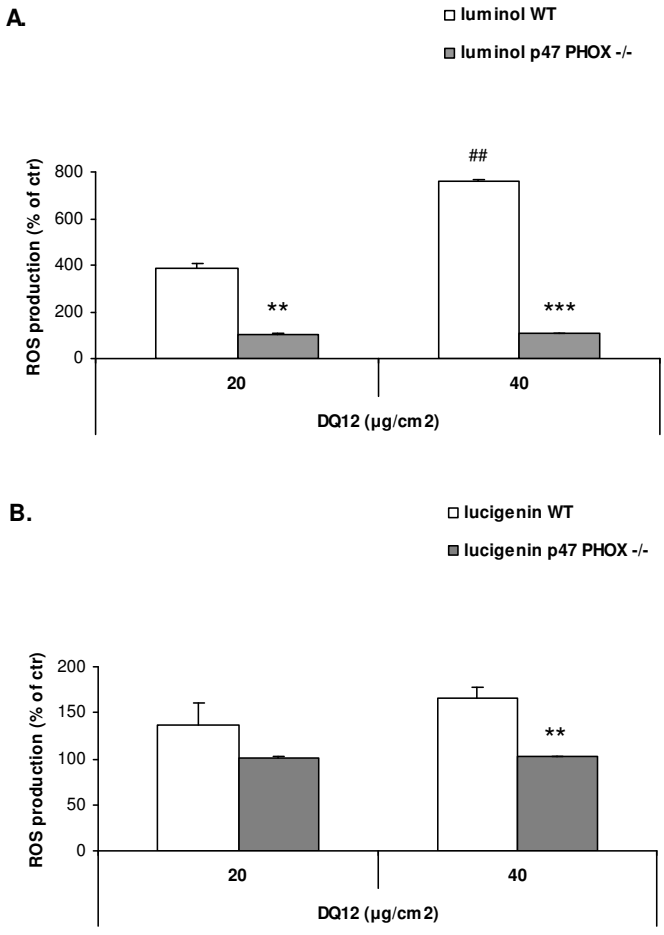
Statistical analysis

Data from the *in vivo* experiments are expressed as mean ± SEM and represent 5 animals per treatment group. The *in vitro* data are shown as mean ± SEM from n = 3 independent experiments. For statistical evaluation, SPSS version 15 for Windows was used. Treatment-related differences were investigated by one-way ANOVA. Multiple comparisons where evaluated using Tukey's method. Difference were considered to be statistically significant when $p < 0.05$.

4.3 Results

Oxidative burst from WT or p47$^{phox-/-}$ neutrophils

Chemiluminescence analysis confirmed that the neutrophils obtained from p47$^{phox-/-}$ mice are incapable of oxidative burst in response to the NADPH oxidase activator PMA, in contrast to the neutrophils from WT mice (Data not shown). We also determined whether the neutrophils from the different mouse backgrounds responded differently to quartz treatment (Figure 1). DQ12 quartz particles caused ROS generation by neutrophils from WT mice, while this response was significantly impaired in the neutrophils from the p47$^{phox-/-}$ animals. This confirms that ROS generation by quartz-treated neutrophils occurs in a NADPH oxidase-dependent manner and also provides strong support for the applicability of the p47$^{phox-/-}$ mouse model to study its role in quartz-induced lung diseases.



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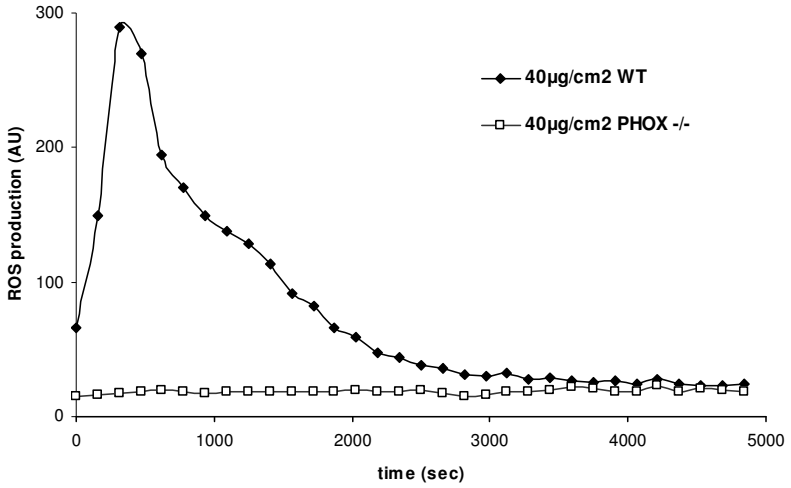


Figure1.ROS generation from quartz treated bone marrow neutrophils from WT or p47$^{phox-/-}$ mice. PMN derived from WT and p47$^{phox-/-}$ mice were treated with 20 or 40 µg/cm^2 DQ12 quartz, and ROS formation was determined by luminol- and lucigenin-enhanced chemiluminescence. Data are shown in figure 1A and 1B, respectively and represent mean ± SEM of three independent experiments. * = p<0.05, ** = p<0.01, *** = p<0.001 *vs.* ROS generation in neutrophils from p47$^{phox-/-}$ mice. ## = p<0.01 *vs.* treatment of WT neutrophils with 20 µg/cm^2. A representative curve derived from a lucigenin experiment is shown in Figure 1C.

DNA damage by quartz in A549 cells in mono- or coculture with WT and p47$^{phox-/-}$ neutrophils

Treatment of the A549 cells with H_2O_2, used as positive control, caused a statistically significant induction of DNA strand breaks and specific oxidative DNA damage (Figure 2). A significant induction of oxidative DNA damage was also observed after treatment with DQ12. In the coculture model, significant oxidative DNA damage induction was only observed with WT neutrophils. In coculture with p47$^{phox-/-}$ neutrophils, quartz treatment did not cause significant DNA damage in the A549 cells. These findings indicate that neutrophils significantly contribute to quartz-induced oxidative DNA damage via the NADPH oxidase pathway of ROS generation.

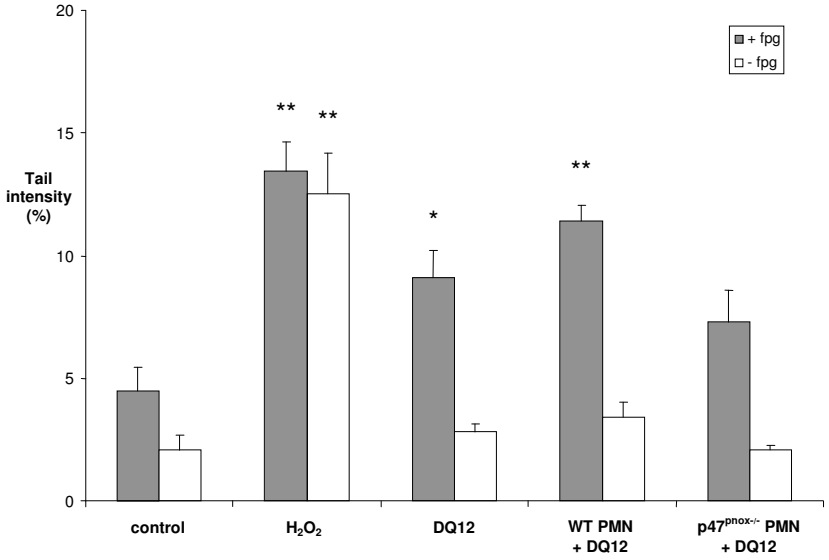


Figure 2. DNA strand breakage and oxidative DNA damage in A549 cells in mono- or coculture with neutrophils from WT or p47$^{phox-/-}$ mice.
DNA strand breakage and specific induction of oxidative DNA damage was quantified by performing the fpg-modified comet assay. Data represent the mean tail intensity ± SEM derived from three independent experiments. * = p<0.05, ** = p<0.01 *vs.* untreated cells (=control).

Lung inflammation in quartz treated WT and p47$^{phox-/-}$ mice

The role of the phagocyte NADPH oxidase was also investigated *in vivo* using both mouse backgrounds. Evaluation of total cells present in BALF showed a marked inflammatory cell influx in response to quartz in both WT and p47$^{phox-/-}$ mice (Table 1).

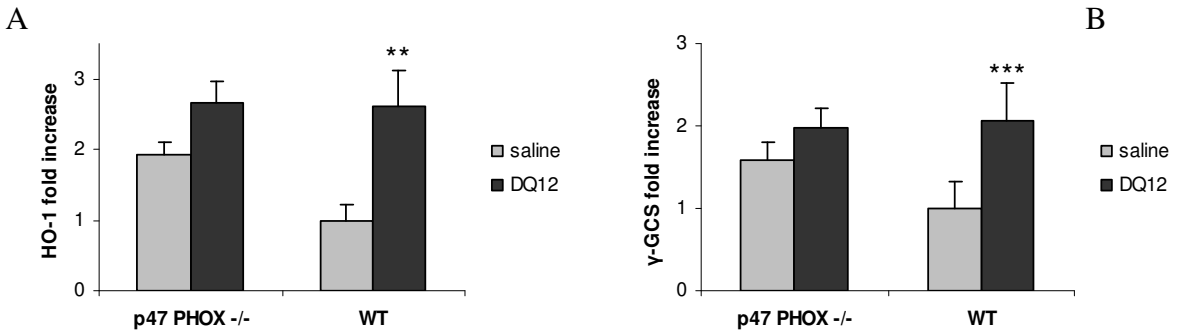
BALF	WT control	WT DQ12	p47$^{phox\ -/-}$ control	p47$^{phox\ -/-}$ DQ12
Total cells (x10^5)	1.30 ± 0.32	7.27 ± 0.57***	3.00 ± 0.79	8.32 ± 1.35**
% neutrophils	0.3 ± 0.24	78.43 ± 4.9***	7.28 ± 4.68	75.02 ± 2.51***
% macrophages	73.98 ± 16.67	18.80 ± 3.52**	84.86 ± 3.89	18.76 ± 3.48***
% eosinophils	0.05 ± 0.05	0.88 ± 0.88	0.20 ± 0.15	0.00 ± 0.00
% lymphocytes	0.05 ± 0.05	0.43 ± 0.36	1.44 ± 0.67	1.32 ± 0.53
% epithelial cells	25.63 ± 16.78	1.50 ± 0.78	6.28 ± 2.36	4.92 ± 1.80

Table 1. Differential analysis of cells obtained by bronchoalveolar lavage (BAL). BAL was performed for 5 animals of each of the four treatment groups. Data are shown as mean ± SEM. Statistically significant differences are indicated as ** = p<0.01 and *** = p<0.001).

Differential analysis indicated that the influx was neutrophil-dominated and remarkably similar for both mouse strains. No significant change in absolute macrophage number was found, confirming that the observed differences in % macrophages reflected its inverse association with the % neutrophils. In the saline-treated p47$^{phox-/-}$ mice a mildly increased inflammatory status was notable unlike in saline-instilled WT mice, indicative of potential constitutive differences. However, the observed differences did not reach a statistical significance.

Oxidative stress and DNA damage in quartz treated WT and p47$^{phox-/-}$ mice

To assess pulmonary oxidative stress after quartz exposure, the mRNA expression of



HO-1 and γ-GCS was measured in lung tissue homogenate (shown in Figure 3A and 3B respectively). Quartz exposure induced a statistically significant up-regulation of the expression of both genes in the lungs of the WT mice, but not in the p47$^{phox-/-}$ animals. These results show that, despite triggering a similar acute inflammatory response characterised by the specific influx of neutrophils, quartz exposure causes less oxidative stress in the lungs of the p47$^{phox-/-}$ mice than in the WT mice. Oxidative DNA damage induction by quartz in the p47$^{phox-/-}$ and WT mice was investigated by the fpg-modified comet assay (Figure 3C). Clear differences in comet tail length could be determined in the absence *vs.* presence of the fpg-enzyme, indicating that oxidative DNA damage could be specifically evaluated in lung tissue. However, no increases could be detected after quartz exposure in either mouse model. These data suggest that, although quartz caused inflammation (and oxidative stress in WT mice), the extent of this may not have been sufficient to cause detectable DNA lesions.

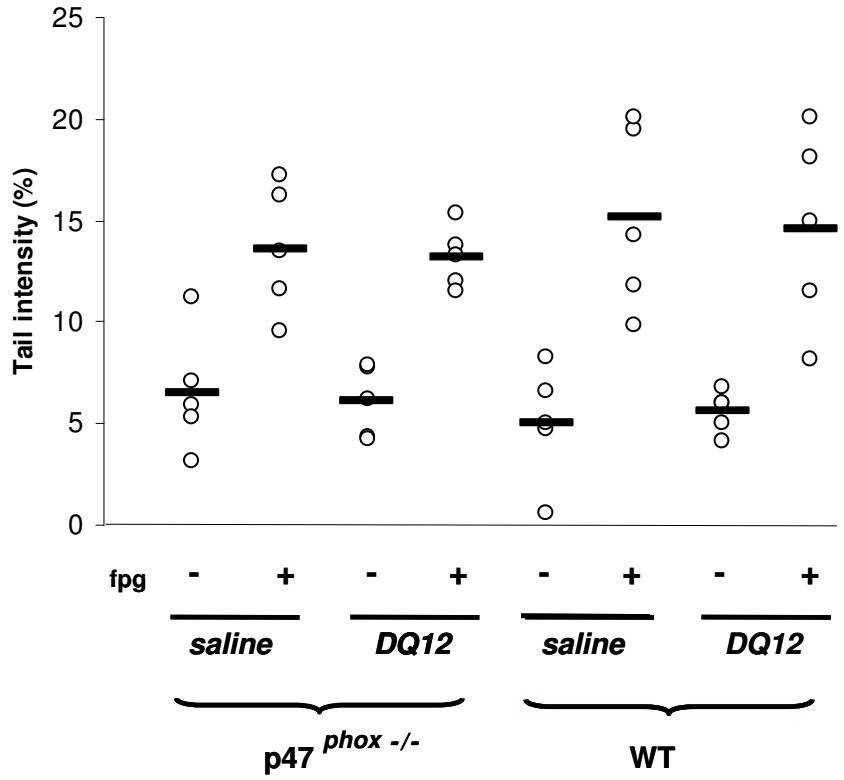


Figure 3. Oxidative stress and DNA damage in the lungs of quartz exposed WT and p47$^{phox-/-}$ mice. Lungs from 5 animals per group were analysed for the mRNA expression of HO-1 (A) and γ-GCS (B) by qRT-PCR, and for (oxidative) DNA damage by the fpg-modified comet assay. mRNA data are expressed as HPRT-adjusted mean ± SEM of the fold induction compared to saline treated WT animals, ** = p<0.01 and *** = p<0.001 *vs.* saline treated p47$^{phox-/-}$ mice. Figure 3C shows individual tail intensity values in the absence or presence of fpg enzyme for each animal, while the horizontal bars indicate mean values for all individual treatment groups.

DNA base excision repair expression in the lungs of quartz treated WT or p47$^{phox-/-}$ mice

To determine potential effects of the quartz-exposure on DNA damage repair, the mRNA expression of the DNA BER enzymes OGG1, APE-1, DNA Polβ and XRCC1 was determined (Figure 4). As shown in the figure, quartz exposure did not significantly modify the expression of any of these genes in either p47$^{phox-/-}$ or WT mice. Immunohistochemistry was used in addition to localise the protein expression of the BER glycosylase OGG1. Representative lung sections are shown in figure 5. In the WT as well as the p47$^{phox-/-}$ mice an increased staining was observed after quartz-exposure. Immunoreactivity tended to be somewhat stronger in the saline-treated p47$^{phox-/-}$ animals, when compared to the saline-treated WT mice. However,

staining appeared to be merely limited to cytoplasma - and occasionally to nuclei - of neutrophils and macrophages, and was not observed in alveolar epithelial type I and type II cells.

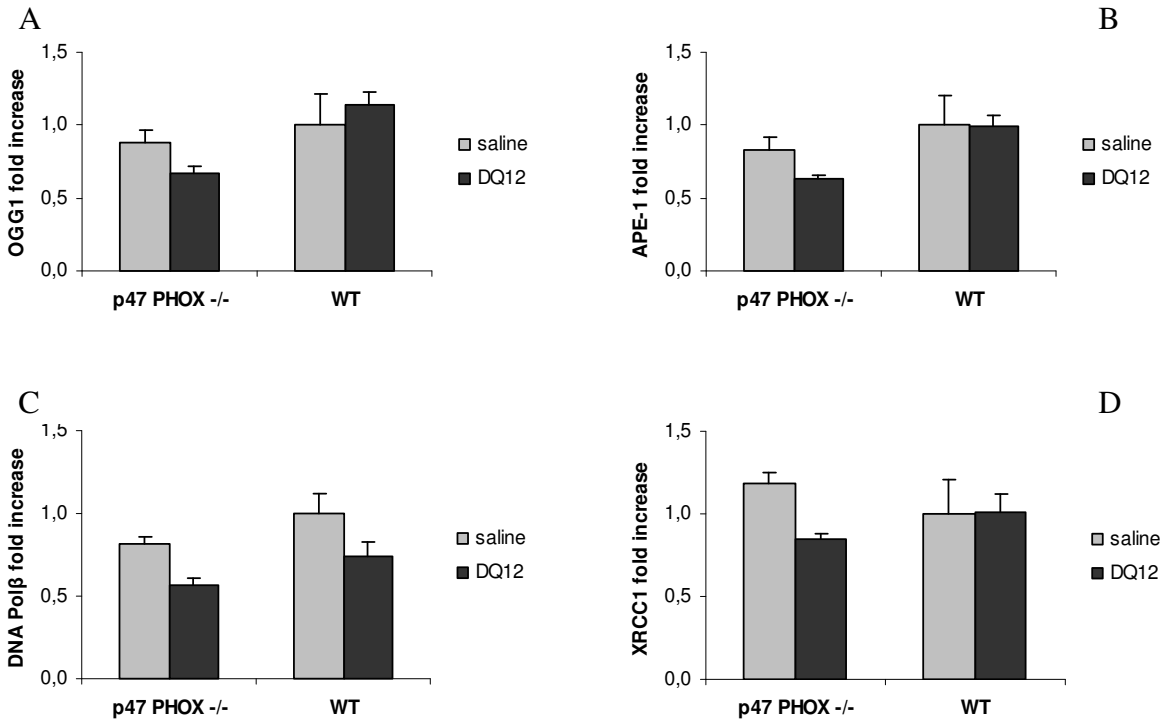


Figure 4. mRNA expression of the DNA base excision repair genes OGG1, APE-1, DNA Polβ and XRCC1 in whole lungs tissues of quartz exposed WT and $p47^{phox-/-}$ mice. Quantitative RT-PCR was used to measure mRNA expression of OGG1 (A), APE-1 (B), DNA Polβ (C) and XRCC1 (D) in lung homogenates of 5 animals per treatment group. Data are expressed as the HPRT-corrected fold induction compared to saline-instilled wildtype animals and shown as mean ± SEM.

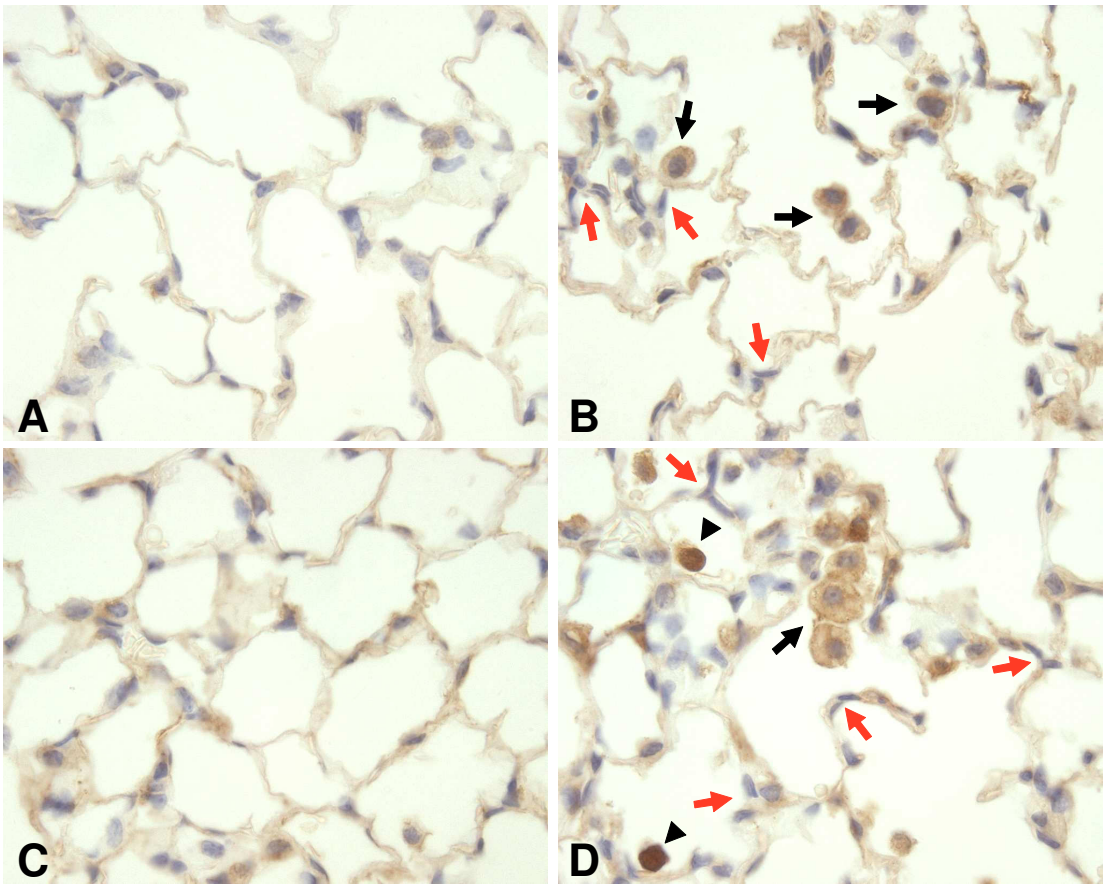


Figure 5. Representative images of OGG1 staining in lung sections, obtained from a saline-instilled WT mouse (A), a DQ12-treated WT mouse (B), a saline-instilled p47$^{phox-/-}$ mouse (C) and a DQ12-treated p47$^{phox-/-}$ mouse (D). Expression of the BER protein OGG1 was determined in mouse lung tissue sections by immunohistochemical analysis. OGG1 protein is visualised by the dark brown staining while the blue/purple staining shows the cell nuclei. Red arrows indicate alveolar epithelial type II cells, while black arrows and black arrowheads point out macrophages and neutrophils, respectively. Original magnification: 1000 ×.

4.4 Discussion

Acute exposure to quartz via pharyngeal aspiration was found to cause a marked pulmonary inflammation in WT and p47$^{phox-/-}$ mouse lungs, characterised by a highly specific influx of neutrophils. Our results are in accordance with previously published findings in rats (15, 16) and mice (30, 31). In fact, quartz has also been used as a positive control in particle toxicology studies in mice (e.g. 32). Despite the clear induction of an inflammatory response in both mouse background models, in the p47$^{phox-/-}$ animals quartz induced less oxidative stress, as indicated by the expression of the oxidative stress markers HO-1 and γ-GCS (9,11-13). The extent of inflammation in terms of neutrophil recruitment appeared to be rather similar in response to the quartz exposure for both mouse models, which indicates that phagocyte-produced ROS do not play a role in the instigation of the acute inflammatory cascade induced by toxic particles. On the one hand, our data suggest that the initiation of inflammation in both mouse strains is predominantly driven by the high bolus dose of quartz administered through pharyngeal aspiration, irrespective of the NADPH oxidase status of alveolar macrophages. Investigation of the role of these resident phagocytes in the initial induction of inflammation by quartz using the p47$^{phox-/-}$ mouse model would necessitate an inhalation exposure study. On the other hand, our findings shows that the induction of oxidative stress as observed in the lung tissue of wildtype mice after 24 hours reflects, at least in part, pro-oxidative effects of activated neutrophils.

In the current *in vivo* study, no quartz-related induction of oxidative DNA damage could be detected in lung tissue of WT or p47$^{phox-/-}$ mice. Also, no differences between p47$^{phox-/-}$ and WT mice were found. Contradicting these findings, in one of our earlier studies, enhanced DNA damage was found in rat lungs after intratracheal instillation at a 10-fold lower quartz dose (10 mg/kg) (25). For the observed differences several explanations may be given: While in the rat study DNA damage was specifically measured in isolated lung epithelial cells, in current investigations whole lung tissue was used. Under baseline conditions, the rodent lung contains around 50 different cell types, and therefore the failure to detect increased DNA damage could possibly be due to dilution effects. However, the observed differences may also reflect species differences in antioxidant defence, which have been

discussed in relation to observed contrasts in tumourigenesis in rats *vs.* mice (33, 34).

With regard to potential antioxidative effects, the significant up-regulation of HO-1 upon quartz exposure in the wildtype mice may be of major relevance. HO-1 is known to have strong anti-oxidative properties and its enhanced protein expression in the lung in response to oxidative stress is widely regarded as a protective mechanism against oxidative tissue injury (35, 36). More specific, in mice, HO-1 has been shown to protect against NOX2-mediated ROS production and oxidative stress (37) and against silica-induced lung injury (12). In a recent *in vitro* study, we found that quartz triggers a rapid increase in HO-1 mRNA expression in rat lung epithelial cells along with oxidative DNA damage induction, suggesting that the up-regulation of this gene represents a potential adaptive response to quartz-induced DNA damage (13). Our current observations in wildtype versus $p47^{phox-/-}$ mice indicate that HO-1 induction in quartz-exposed lung is, at least in part, caused by neutrophil-derived ROS and thereby may protect this organ from a significant steady-state increase in oxidative DNA damage. Similarly, our current findings indicate a potential role for the GSH pathway, witnessed by the enhanced expression of the rate-limiting enzyme γ-GCS (9) after acute quartz exposure.

A further possible explanation for the currently observed absence of oxidative DNA damage induction may be that the mice are highly efficient in oxidative DNA damage repair, which then also could explain the aforementioned notion on the contrast in quartz-induced tumourigenesis in mouse and rat. To address for potential contrasting effects of DNA damage repair in the different mouse models we measured the mRNA expression of OGG1, APE-1, DNA Polβ and XRCC1, shown to be important for the effective repair of oxidative DNA lesions and hence also proposed as biomarkers of oxidative stress (38,39,30,41,42). However, we could not detect any significant change in the mRNA expression of any out of these four DNA repair genes. Enhanced expression of OGG1 mRNA has been previously shown in association with X-ray-induced oxidative DNA damage in mouse lungs (28), and after challenge with diesel exhaust particles in the lungs of rats (43) and mice (44). Because of the absence of such observation in our current study, we also determined its expression on the protein level by immunohistochemistry. These analyses indicated an increased immunoreactivity for OGG1 protein in response to quartz treatment in both mouse backgrounds. Importantly however, staining appeared to be limited to the

phagocytes present in the alveolar lumen. Despite the high dose of silica administered, the protein expression of OGG1 was found to be unaffected in structural lung cells including alveolar epithelial type II cells. These observations are of importance as the alveolar type II cell is considered as the major target cell type in quartz-induced tumourigenesis in rodents (4, 33). It will also be interesting however, to further evaluate the potential role of BER expression changes in phagocytes. In particular this might be of relevance in relation to the discussed role of macrophage apoptosis in quartz-induced lung disease (45). Notably, in the current study, the lungs in which mRNA expression of OGG1 and the other BER genes were determined were subjected to bronchoalveolar lavage. Hence, marked mRNA expression changes in macrophages after quartz treatment would unlikely have been detected.

In contrast to our *in vivo* findings, our parallel *in vitro* studies showed that quartz particles at high concentrations are capable of eliciting oxidative DNA damage in lung epithelial cells, in concordance with previous findings (8, 13, 46). Recent *in vitro* studies in our laboratory led us to suggest that this damage can result at least in part from ROS originating from mitochondria of quartz-treated cells via a yet-to-be-elucidated mechanism (46). In previous studies we have also shown that neutrophils upon activation with PMA, can damage the DNA of lung epithelial cells *in vitro* (47.) Now in addition, we show that oxidative DNA damage induced by quartz in alveolar epithelial cells cocultured with neutrophils- used as a model to mimic neutrophilic pulmonary inflammation after acute quartz exposure- is NADPH oxidase dependent. Notably, quartz-induced DNA damage in the A549 cells was not significantly enhanced in the presence of $p47^{phox-/-}$ neutrophils, while quartz alone caused significant DNA damage. This difference may relate to potential protective effects of antioxidants which are known to be abundantly present in neutrophils. However, it may also be due to the mere fact that the silica particles were less accessible to the epithelial monolayer in the cocultures because of the physical presence of the neutrophils. As such one can also assume that similar antioxidant or dosimetry differences will have reduced the level of DNA damage induction in the cocultures containing wildtype neutrophils. Taken together, our findings clearly show that neutrophils contribute to quartz-induced genotoxicity through the induction of oxidative DNA lesions *in vitro*. The specific role of NADPH oxidase activation by quartz and resulting ROS generation could be demonstrated by comparative

chemiluminescence analysis of quartz-treated neutrophils from wildtype or p47$^{phox-/-}$ mice. These findings also support recent studies that revealed the importance of this phagocyte complex in quartz-induced activation of the inflammasome pathway and resulting inflammation (48, 49).

In summary, in the present study we demonstrate that neutrophil-derived ROS significantly contribute to oxidative stress in acute quartz-exposed mouse lungs. In addition we could confirm the hypothesised importance of NADPH oxidase-mediated ROS formation in quartz genotoxicity *in vitro*, however not *in vivo*. This apparent paradox is most likely explained by dose related differences, both in terms of the quartz and the neutrophils in the cocultures. While the *in vitro* experiments were performed with 100 µg/cm^2 quartz and a 2:1 ratio of neutrophils to epithelial cells, the *in vivo* conditions reflect an approximate dose of 5 to 10 µg quartz per cm^2 rat lung epithelial surface, and, based on the bronchoalveolar lavage findings, an estimated neutrophil to epithelial cell ratio of about 1:10. Oxidative stress responses, whether resulting from direct quartz particle properties and/or from quartz-activated neutrophils, are considered to occur in a hierarchical fashion in different tiers (50). It seems therefore likely that, in contrast to our *in vitro* experiments, neutrophil-derived-ROS *in vivo* did not act on the highest tier, involving DNA damage, while lower tier effects were reflected by the induction of sensitive antioxidant pathways including γ-GCS and HO-1 (40) and pulmonary inflammation. However, in view of our observed *in vitro* effects and the knowledge that the used A549 cell line is highly robust to oxidative stress, it cannot be fully ruled out that neutrophil-dependent DNA damage and subsequent mutagenesis will be detectable in more sensitive mouse models. A relevant approach would be to use the p47$^{phox-/-}$ model in combination with DNA damage repair deficient mice, with inclusion of specific evaluation of lung epithelial cells. Such investigation should also take into account the role of the persistence of inflammation and associated shifts in the pattern of phagocyte involvement, i.e. neutrophils and macrophages in relation to differences in their specific oxidative stress-relevant constituents such as myeloperoxidase and nitric oxide synthase (51).

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Chapter 5

5. Summary and general discussion

Epidemiological studies revealed an association between particle exposure, cardiovascular diseases and lung cancer (1, 2).

Cancer is a multistep process and includes genetic alterations like activation of oncogenes, inactivation of tumor suppressor genes, and deficient cell cycle control and/or DNA repair processes (3-5). DNA damage is considered as an essential feature in the initiation of cancer. One of the most prominent and investigated DNA lesion is the oxidative DNA damage 8-OHdG. Several *in vivo* as well as *in vitro* studies in the field of particle research showed, that exposure to particles can cause oxidative DNA damage (6-9). Oxidative DNA damage can be seen as the consequence of cellular oxidative stress, which describes a disturbance in the balance of oxidants and antioxidants (10).

The ability of particles to cause oxidative DNA damage has led to the introduction of the terms primary and secondary genotoxicity. In this regard, primary genotoxicity arises from ROS/RNS generation by intrinsic particle characteristics like surface properties or by the interaction of the particles with cellular compartments. Secondary genotoxicity includes the generation of ROS/RNS during particle-elicited inflammation (11).

The **aim** of this thesis was to investigate and elucidate the relationship between exposure, particle characteristics, inflammation, oxidative stress, oxidative DNA damage and its associated repair. Three independent studies were performed, which were different in the choice of particulate matter and experimental model as well as with regard to relevant endpoints of cytotoxicity, inflammation, oxidative stress/response and DNA damage/repair.

In **chapter 2** (study 1) the H_2O_2-dependent oxidant 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 at four contrasting locations in the UK was determined by electron spin resonance (ESR). Additionally, we determined in the A549 human lung epithelial cell line, the cytotoxicity of samples by LDH assay, and interleukin-8 (IL-8) release as an indicator of their inflammatory potency. Oxidative DNA damage was measured by the formamido-pyrimidine-glycosylase (fpg)-modified comet assay. The study design

allowed a more detailed evaluation of size-effects in combination with sampling locations differing in traffic background levels.

We demonstrated that the oxidant generating capacity of PM depends rather on the sampling site than on the size of the particles. PM samples collected at sites with a high traffic background showed higher levels of oxidant generation than samples of the same size from locations with less or no traffic background. The same trends were found for the induction of cytotoxicity (measured as the release of LDH) and oxidative DNA damage (measured by fpg-comet assay). The data confirm that traffic is an important source of toxic particles. Interestingly, the smallest size fraction (< 0.5 µm) showed the strongest impact on the release of the pro-inflammatory cytokine IL-8. This is in contrast to former studies with ambient PM samples, which showed that the coarse fraction of PM causes stronger release of IL-8 than the fine fraction (12, 13). We based our treatment on equal mass basis and therefore our results are in agreement with the finding, that the surface area is a main contributor to the initiation of pro-inflammatory processes (14, 15).

In **chapter 3** (study 2) we were interested, if short-time inhalation of spark-discharge generated carbon nanoparticles can lead to cytotoxicity, inflammation, oxidative stress, oxidative DNA damage and its repair in mice as well as rats. These particles represent a model of combustion-derived nanoparticles in ambient air. Most of the available studies used complex mixtures of particles containing metals and organics (16). After exposure to the spark-generated carbon nanoparticles no effects were measured in all investigated endpoints. Expression analysis of oxidative stress response, DNA BER repair genes and oxidative DNA damage were performed with whole lung tissue of the lungs of mice and with alveolar epithelial cells isolated from the rat lung. The similarity of findings between (a) both rodent models and (b) between whole lung tissue and alveolar epithelial cells strengthen our results.

The study presented in **chapter 4** (study 3) was done to reveal the role of the phagocyte specific NADPH-oxidase generated ROS during particle-elicited inflammation. We observed an influx of inflammatory cells into the lung after pharyngeal aspiration of DQ12 in the WT as well KO mice. The inflammatory response was remarkedly similar in both mouse strains, suggesting that the initial pro-inflammatory response is independent of NADPH-oxidase, which is maybe an effect of the high bolus dose of DQ12.

However, we demonstrated that neutrophil-derived ROS significantly contribute to oxidative stress in acute quartz-exposed mouse lungs. Moreover, we could confirm the hypothesised importance of NADPH oxidase-mediated ROS formation in quartz genotoxicity *in vitro* in a co-culture model of isolated bone-marrow derived neutrophils and A549 cells, however not *in vivo*.

For particle-related genotoxicity two major types of DNA damage can be considered, on the one hand DNA damage related to the attack of ROS and on the other hand the formation of bulky DNA adducts by organics as PAH. To distinguish the particle related effects and its relevance in risk assessment purposes, the concept of primary and secondary pathways of genotoxicity has been introduced (17, 18).

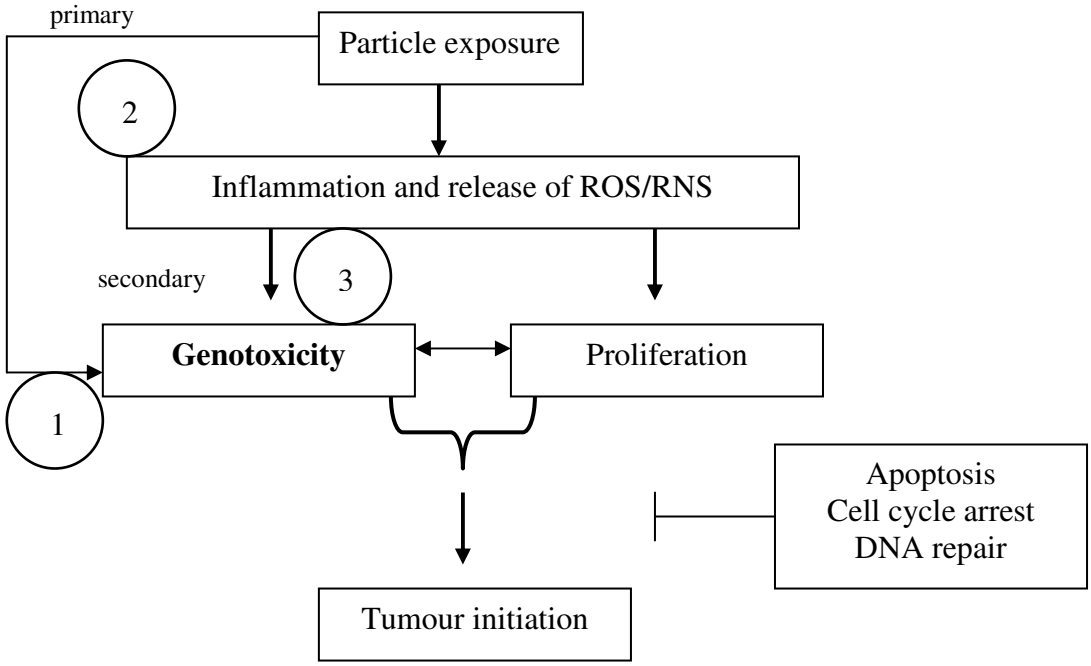

Figure 1. Pathways of primary and secondary genotoxicity as implicated in particle carcinogenesis. In Particle induced carcinogensis, particles are considered to impact both on genotoxicity and cell proliferation. The focus of this thesis was on genotoxicity, and two modes of actions of particles can be considered. Secondary genotoxicity is considered to be driven by ROS generated during particle-elicited inflammation. Primary genotoxicity implies an induction of oxidative DNA damage elicited by intrinsic particle properties. The circles indicate, how the particular studies of this thesis fit into this concept.
(modified from Schins & Knaapen, 2007, 19)

In figure 1 the concept of primary and secondary genotoxicity is presented. As it can be seen, the respective studies presented in this thesis are each within the framework of this concept. Particle research associated genotoxicity studies are highly relevant since epidemiological studies found a relation between ambient PM

exposure and the risk of lung cancer (2). Many studies postulated that particle-bound substances as transitions metals and organics as PAH determine the toxicity of ambient PM (20, 21). Moreover, the surface area of particles has been considered of importance specifically for nanoparticles. The design of the presented **study 1** allowed a more detailed evaluation of potential size distribution-dependency in relation to the origin of these particles. As we pointed out, the sampling location was more important than particle size. The samples collected from traffic-rich locations and therefore likely the impact of particle-bound transitions metals or organics is higher in relation to the induction of toxicity effects than specific size effects. However, for the release of IL-8 it appeared that particle size and surface area were more important.

To investigate the effects of pure carbon nanoparticles, a short-time inhalation study was performed in mice and rats. No effects were measured for all investigated endpoints (**study 2**). It has been postulated that genotoxicity by particles is considered to involve a threshold, which value is set by the exposure concentration, which will trigger inflammation and overwhelm antioxidant and DNA damage repair capacities in the lung (17). Oxidative stress responses, whether of primary or secondary origin are occurring in a hierarchical fashion. Li and co-workers introduced the model of different tiers for the role of oxidative stress in the development of particle toxicity (22). This model may also be adapted for oxidative DNA damage. In this model the lowest tier is reflected by the induction of antioxidant pathways. Within this model the results from the size-fractionated ambient PM *in vitro* study (**study 1**) can be ranked in the highest tier. In contrast to the results of **study 2**, which have to be ranked into the lowest tier (no effects). The in vivo results of **study 3** can be related to the middle tier. This is reflected by the induction of the antioxidant genes HO-1 and γ-GSC and the influx of neutrophils. In this study we found contrasting results between our *in vivo* and *in vitro* approach. The importance of the NADPH-oxidase mediated ROS formation in quartz related genotoxicity was only confirmed *in vitro* not *in vivo*. Thus, the *in vitro* approach reflected tier 3 effects (oxidative DNA damage responses).

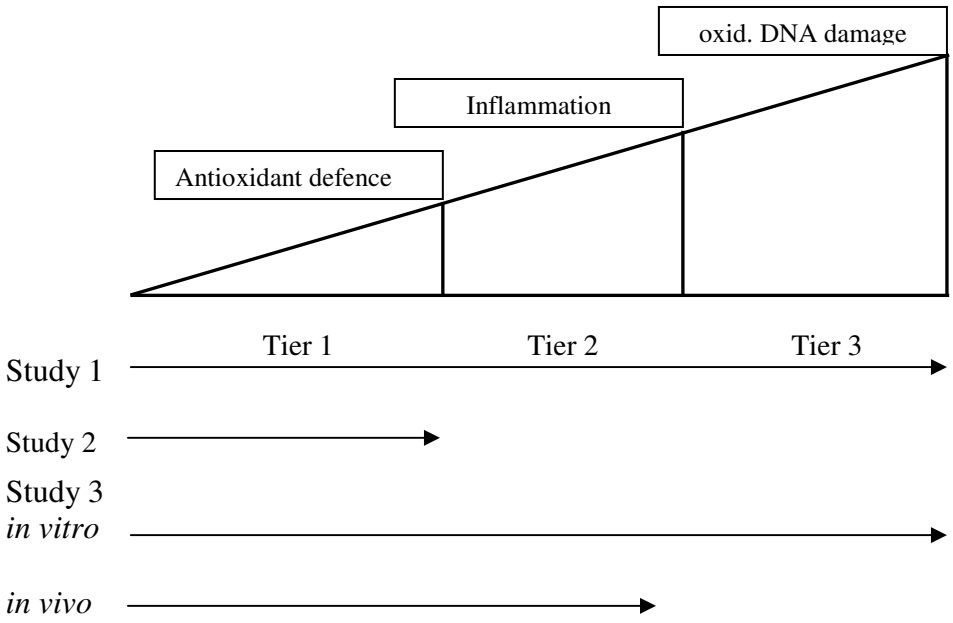

Figure 2. Classification of the findings of the three studies of this thesis in the framework of the hierarchical oxidative stress model (adapted from Li and Nel, 2006, 22).

This could, as discussed in **chapter 4**, most likely explained by dose related effects between *in vitro* and *in vivo* experiments. This paradox leads to a general problem in particle toxicity. On the one hand mechanistic studies have to be done, to reveal possible toxic endpoints after exposure to particles. However, these studies are mostly using high-doses of a certain particle. In view of our observed *in vitro* effects and the knowledge that the used A549 cell line is highly robust to oxidative stress, it cannot be fully ruled out that neutrophil-dependent DNA damage and subsequent mutagenesis can occur *in vivo*.

For risk assessment these findings of high-exposure situations can only give a hint (= hazard) regarding the actual situation in human exposure settings (= dose). Therefore more advanced approaches have to find their way in the field of particle research. The use of specific knock-out models is an interesting approach. For example, in the case of oxidative DNA damage and DNA repair the use of oxidative DNA damage deficient mice (e.g. Ogg1-CSB double knockout mice) can reveal the importance of DNA repair of oxidative DNA damage after particle exposure. Concerning possible compensation by DNA repair pathways to particle induced

oxidative DNA damage also specific activity assays for certain DNA repair enzymes are a promising approach.

A further interesting approach of an integrated test design can be the application of the ESR technique for monitoring online the oxidant-generating capacity in ambient PM samples: As explained in **chapter 2** of this thesis, ROS is highly correlated to all measured toxicological endpoints, and therefore may predict potential adverse health effects.

Nowadays public concern is shifting from the “giant” particles towards the “dwarf” particles. Nanotoxicology is a relatively new field and it is facing unexpected new particle properties (e.g. quantum effects). Nanoparticles are able to enter cells and can interact directly with cellular compartments, such as mitochondria (23) or the nucleus (24). Besides entering cells, nanoparticles are also able to translocate into the blood circulation and become accumulated in several organs. Interestingly, nanoparticles can enter the brain directly via the olfactory nerve (25). These findings show the complexity of possible effects of nanoparticles. Ongoing work has also to focus on the development of verified test methods, which can give reliable results about the toxicity of nanoparticles to various oranges and tissue.

Taken together, particle research remains an important field of science, especially since the industry currently concentrates on the development of new “nano”-approaches.

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6. Zusammenfassung

Ziel dieser Arbeit war die Untersuchung der Mechanismen die zu einer Induktion von oxidativen DNA-Schäden nach Partikelexposition führen können. In diesem Zusammenhang wird zwischen partikel-induzierter (primärer) und Entzündungs-induzierter (sekundärer) Genotoxizität unterschieden. Es wurden drei Studien mit unterschiedlicher Fragestellung, Partikelwahl und Dosimetrie durchgeführt.

In Studie 1 wurde die humane Lungenzelllinie A549 mit größen-fraktionierten Außenluftpartikeln, gesammelt an Orten mit unterschiedlichem Verkehrsaufkommen belastet. Es wurden signifikante Unterschiede in Bezug auf Hydroxylradikal-Bildungsfähigkeit, Zytotoxizität, Interleukin-8-Ausschüttung und oxidativen DNA-Schäden festgestellt. Dabei zeigte sich, dass die Quelle der Partikel eine größere Bedeutung als die Größenfraktion hat.

Studie 2 zeigte, dass eine Kurzzeitinhalation von Kohlenstoffnanopartikeln (~150 µg/cm³) weder zu einer pulmonalen Entzündung in beiden Nagermodellen (Maus und Ratte) noch zu einer Erhöhung von oxidativen DNA-Schäden bzw. mRNA Expression von DNA Reparaturgenen in der Gesamtlunge (Maus) oder in alveolaren Epithelzellen (Ratte) führte.

Studie 3 zeigte, dass obwohl die pharyngeale Aspiration von Quarz zu einer signifikanten pulmonalen Entzündung in WT- und $p47^{phox-/-}$-Mäusen führt, $p47^{phox-/-}$-Mäuse reduziertem oxidativem Stress ausgesetzt sind. Dennoch konnte keine oxidative DNA-Schädigung bzw. erhöhte mRNA-Expression von DNA Reparatur-genen in der Lunge Quarz-behandelter WT- oder KO-Mäuse gemessen werden. Dagegen wurde in Kokultur-Experimenten die Bedeutung der NADPH-Oxidase-abhängigen ROS-Bildung von neutrophilen Granulozyten nach DQ12-Belastung für die Induktion oxidativer DNA-Schäden in Lungenepithelzellen gezeigt.

Die Ergebnisse dieser Arbeit zeigen, dass nach Exposition mit hohen Partikel-konzentrationen eine Entzündungsreaktion entscheidend für die Ausbildung geno-toxischer Effekte ist. Die durch NADPH-Oxidase-Aktivierung freigesetzten ROS der neutrophilen Granulozyten scheinen für die Ausbildung der Genotoxizität von Bedeutung zu sein. Neben der Entzündung trägt die chemische Zusammensetzung der Partikel (Metalle, polyaromatische Kohlenwasserstoffe, Partikeloberflächen) maßgeblich zur Ausbildung gesundheitsschädigender Effekte bei.

7. Summary

The aim of this thesis was to elucidate the mechanisms leading to oxidative DNA damage after particle exposure. In this regard one has to distinguish between primary (particle-driven) and secondary (inflammation-driven) genotoxicity. Three independent studies were performed, which were different in the choice of particulate matter and experimental set-up.

In study 1 the human lung epithelial cell line A549 was exposed to size-fractionated ambient PM samples, collected from locations with contrasting traffic profiles. Significant differences were found in oxidant-generating capacity, cytotoxicity, interleukin-8 release and oxidative DNA damage. Effects were found to depend more on the sampling site than on the size fraction. The data confirm that traffic is an important factor for the toxic potential of ambient PM samples. In addition, significant correlations were observed between the oxidant generating potential and all toxicological endpoints.

Study 2 revealed, that short-time inhalation of carbonaceous nanoparticles (~150 µg/cm^3) leads neither to pulmonary inflammation nor to an induction of oxidative DNA damage and associated induction of DNA repair genes in whole lung tissue of mouse or alveolar epithelial cells isolated from rats.

Study 3 was performed to reveal the role of reactive oxygen species (ROS) from the phagocyte NADPH-oxidase during particle-elicited inflammation. Although an influx of inflammatory cells into the lung of wildtype as well as p47$^{phox-/-}$ mice was observed after pharyngeal aspiration of quartz particles, oxidative stress appeared only in wildtype mice. No effects were found regarding oxidative DNA damage and expression of DNA repair genes. In contrast, the importance of NADPH oxidase-mediated ROS formation in quartz induced genotoxicity was shown in a coculture model of bone-marrow derived neutrophils and A549 cells.

The results of this thesis showed, that pulmonary inflammation is of major importance for the induction of oxidative DNA damage after particle exposure. NADPH-oxidase generated ROS by neutrophilic granulocytes seems to contribute to genotoxic effects after particle exposure. In addition to the pulmonary inflammation the chemical composition (transition metals, PAH, particle surface properties) of ambient particles seems to play an important role.

Curriculum Vitae

Anton Wessels

06.01.1978, Papenburg

Professional experience

05/05 – 12/09 **Institut für umweltmedizinische Forschung,** Research Associate
Particle Research Group, Düsseldorf, Germany

10/04 - 12/04 **University of Bristol,** School of Biological Sciences, Bristol, UK
Internship at the department of Environmental & Evolutionary Physiology and Biochemistry

Education

05/05 - today **PhD thesis**: "Mechanisms of genotoxicity of inhalable particles: *in vitro* & *in vivo* investigations"
Institut für umweltmedizinische Forschung, Particle Research Group, Heinrich-Heine University of Düsseldorf, Germany

07/07 - today **Postgraduate Education in Toxicology**
Participation in several courses

12/06 - 09/09 **Graduate school** GRK 1427: “Food constituents as triggers of nuclear receptor-mediated intestinal signalling”, Heinrich-Heine University of Düsseldorf, Germany

10/98 - 09/04 **Master in Biological Sciences**
University of Osnabrück, Germany

11/03 – 09/04 **Department of Animal Physiology,** University of Osnabrück, Germany
Master thesis: "Interactions of the chitin synthase of *manduca sexta* with cellular proteins"

08/90 - 06/98 **High school**, Papenburg, Germany
Abitur (equiv. to A-levels)

Awards

10/07 **Eurotox Young Scientist Award**
“Nanoparticle-induced inflammation and its effect on the base excision repair pathway in the lung”,
44th congress of the European Societies of Toxicology (Eurotox), October 2007, Amsterdam, The Netherlands.

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.
Ferner versichere ich, dass ich weder an der Heinrich-Heine-Universität Düsseldorf noch an einer anderen Universität versucht habe, diese Doktorarbeit einzureichen.
Ebenso habe ich bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 07.12.2009

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Anton Wessels