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REVIEW ARTICLE

Role of oxidative stress in carbon nanotube-generated health effects

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Abstract The development of products containing carbon nanotubes (CNTs) is a major achievement of nanotechnology, although concerns regarding risk of toxic effects linger if the hazards associated with these materials are not thoroughly investigated. Exposure to CNTs has been associated with depletion of antioxidants, increased intracellular production of reactive oxygen species and pro-inflammatory signaling in cultured cells with primary function in the immune system as well as epithelial, endothelial and stromal cells. Pre-treatment with antioxidants has been shown to attenuate these effects, indicating a dependency of oxidative stress on cellular responses to CNT exposure. CNT-mediated oxidative stress in cell cultures has been associated with elevated levels of lipid peroxidation products and oxidatively damaged DNA. Investigations of oxidative stress endpoints in animal studies have utilized pulmonary, gastrointestinal, intravenous and intraperitoneal exposure routes, documenting elevated levels of lipid peroxidation products and oxidatively damaged DNA nucleobases especially in the lungs and liver, which to some extent occur concomitantly with altered levels of components in the antioxidant defense system (glutathione, superoxide dismutase or catalase). CNTs are biopersistent high aspect ratio materials, and some are rigid with lengths that lead to frustrated phagocytosis and pleural accumulation. There is accumulating evidence showing that pulmonary exposure to CNTs is associated with fibrosis and neoplastic changes in the lungs, and cardiovascular disease. As oxidative stress and inflammation responses are implicated in the development of these diseases, converging lines of evidence indicate that exposure to CNTs is associated with increased risk of cardiopulmonary diseases through generation of a pro-inflammatory and pro-oxidant milieu in the lungs.

Keywords Atherosclerosis · Carbon nanotubes · Comet assay · Endothelial dysfunction · Genotoxicity · Inflammation · Oxidative stress · Reactive oxygen species · ROS production

Introduction

Carbon nanotubes (CNTs) are fiber-like materials, typically described as manufactured nanomaterials with unique electrical, mechanical and thermal properties. Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are the most widely investigated types of CNTs in nanotoxicology. They may be used in pristine form or as surface-modified structures. There has been substantial interest in the development of CNTs, although the fiber-like structure has alerted toxicologists because of their resemblance to hazardous materials such as asbestos. Despite this, the CNT toxicological studies are few as compared to studies on their development and applications (Fig. 1). An inspection of the

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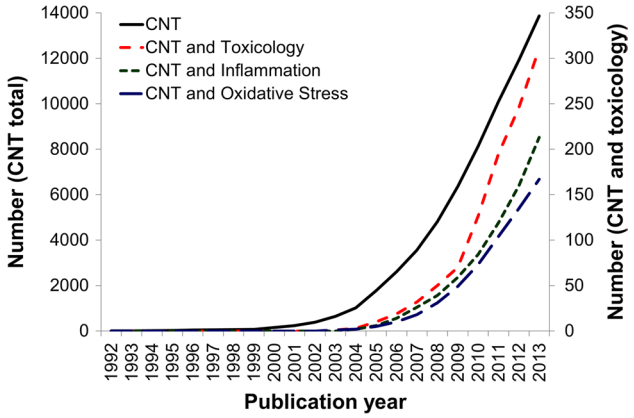

Fig. 1 Accumulation of carbon nanotube publications available on the PubMed database with searches of terms "carbon nanotubes" combined with "toxicity," "inflammation" or "oxidative stress." The *left Y-axis* is the number of all studies on carbon nanotubes, whereas the *right Y-axis* is the combined searches

literature indicates that fibrosis, cancer and cardiovascular disease have received highest priority with regard to the investigated health effects following exposure to CNTs (Donaldson et al. 2006; Shvedova et al. 2012). Inhalation of CNTs in the workplace has been highlighted as relevant mode of exposure (Donaldson et al. 2006; Lam et al. 2006). However, it must be expected that the general population can also be exposed by either dermal or oral routes (Aschberger et al. 2010; Johnston et al. 2010). In addition, the high persistence of CNTs may increase the possibility of accumulation through the food chain (Helland et al. 2007). The risk of disease is probably low for each individual in the general population, whereas it is important on population level because of the large number of exposed individuals. Such potential CNT-associated diseases would be characterized by a long subclinical period before diagnosis, although patients may be alerted by diminished physical performance. In addition, lifestyle factors may render some individuals more susceptible (e.g., people with a high-fat diet and little exercise might be more susceptible to CNT-promoted cardiovascular diseases) or confound CNT exposure–response relationships (e.g., smoking is such a strong lung carcinogen that the additional risk by low-dose CNT exposure may be difficult to assess).

The present review focuses on the role of oxidative stress and inflammation as mechanisms of CNT-generated lung and cardiovascular diseases. Both oxidative stress and inflammation are implicated in the development and progression of cancer and cardiovascular disease (Houghton 2013; Libby et al. 2011). Oxidative stress can occur almost instantly, whereas an inflammatory response takes time to develop. Nevertheless, exposure periods of CNTs are typically sufficiently long (e.g., 24 h in experimental systems), allowing both oxidative stress and inflammation to develop in cells or animals.

Characterization of carbon nanotubes

The types of CNTs differ substantially between studies as do experimental systems and the dispersion protocols that have been applied so far, where inhalation studies are very few. It is clear that the dispersion protocol affects the toxicity of CNTs (Gasser et al. 2012; Mercer et al. 2008). However, biological effects also depend on the fate of CNTs in the organism (e.g., deposition, translocation and biopersistence), which depends on particle characteristics. MWCNTs and SWCNTs are by definition different types of materials. There has been substantial focus on the role of nanomaterial characteristics in studies of toxicity of CNTs. For instance, Madl and Pinkerton (2009) highlighted 15 different potential important characteristics for the biological fate and toxicity (i.e., size, shape and chemical constituents). CNTs are generally biopersistent, and the fiber length is a good predictor of inflammation, especially with frustrated phagocytosis of very long fibers identified as a well-adapted paradigm for pulmonary toxicity (Donaldson et al. 2006). Moreover, fibers can also translocate to the pleura where long length is associated with accumulation (Donaldson et al. 2013). "Short" fibers have been defined as being less than 10–15 μm (Bernstein 2007). Rat macrophages cannot phagocytose fibers longer than 15–20 μm, which is therefore typically defined as "long" fibers (Bernstein 2007; Donaldson et al. 2006). However, the present review indicates that the use of definitions of "long" and "short" CNTs is arbitrary as they often refer to intra-experiment comparisons and not based on the potential hazard of the material.

Samples of CNTs are heterogeneous with respect to the length of individual fibers, and the shape can be needle-like, twisted or tangled. Publications on toxicity of CNTs typically have some information about characteristics of the tested material, although all potentially important characteristics may not have been obtained. The information on CNT length is sometimes only derived from the manufacturer's description of the material in its dry form, whereas the actual size of the CNTs may change during the dispersion. For instance, Kim et al. (2014) reported that a particular type of MWCNT had a length of 20 μm (manufacture's description), whereas transmission electron microscopy (TEM) analysis of 300 individual fibers revealed a heterogeneous distribution (0.068–1.517 μm) with a median length of 0.33 μm. A number of studies contain only information about the range in fiber length, having reported the smallest fibers to be 0.5 μm, whereas the longest fibers were 40–200 μm (Cavallo et al. 2012; Di Giorgio et al.

2011; Lee et al. 2012; Lindberg et al. 2009; Migliore et al. 2010). Another example of size issue comes from the European Union Framework 7 project "ENPRA—Risk Assessment of Engineered Nanoparticles" that originally selected two different samples of MWCNTs as "long" and "short," based on the manufacture's information, but a thorough characterization revealed that the samples had almost the same length of 0.7–4.0 μm (Kermanizadeh et al. 2012, 2013). This indicates that the manufacture's information may not be a reliable source of fiber dimensions and size of CNTs in the actual exposure vehicle. In addition, the large majority of publications indicate that the typical sample of CNTs contains fibers with a median length below 10 μm, indicating that they are best characterized as short fibers. However, fibers with lengths less than 10 μm may also be hazardous. This is demonstrated by observations that fibers with a length of 4 μm are pathogenic to the pleura of mice after direct injection into the cavity (Schinwald et al. 2012).

Another issue that has attracted attention in CNT toxicology is the content of transition metal impurities in the material. The contents of metals and structural defects of MWCNTs have been highlighted as important predictors of genotoxicity and respiratory toxicity (Fubini et al. 2010). Early studies used SWCNTs with a wide difference in residual iron content (0.23 vs 26 % iron by mass) and found biological effects that could be explained by this difference (Kagan et al. 2006). Another early study on animal exposures used CNTs with approximately 25 % of the mass being metal impurities (Lam et al. 2004). However, the majority of later studies have used CNTs with only a few percent of metal impurities, and the extent of structural defects is virtually never reported.

The assessment of oxidative stress endpoints in the studies of the present review has not revealed any CNT characteristics that could be used as common denominators for assessment of effect in terms of genotoxicity and cardiovascular endpoints across publications. Indeed, the complexity of the issue concerning CNT characteristics can be illustrated by assuming that oxidative stress effects can be generated by mainly three different factors (e.g., type, length and iron content). The assessment of these effects and their interactions requires a sample set of eight different CNT samples that do not differ in any other aspects of characteristics (2^3-full factorial design). In comparison, the highest number of CNTs in the publications is typically only 3–4 different materials (Manshian et al. 2013; Murphy et al. 2012; Palomaki et al. 2011; Poland et al. 2008; Pulskamp et al. 2007; Sauer et al. 2014; Yamashita et al. 2010). Supplementary Tables S1 (cell culture studies) and S2 (animal studies) contain information about the characteristics in the publications as well as summaries of effects on oxidative stress, inflammation or disease endpoints. The particle characteristics are obviously important for risk assessment of CNTs, whereas the present review uses a generic approach to focus on hazard identification of CNTs.

CNT uptake in cells

The identification of single CNTs in cells is difficult because they are structures of graphene sheets, indistinguishable from other carbon-based molecules in the cell (Køebler et al. 2014). A new technique based on enhanced-darkfield illumination optics and imaging now makes it possible to visualize single MWCNTs in tissue sections (Mercer et al. 2013a, b). Therefore, it has typically been bundles or large agglomerates of CNTs that have been observed within cells with the present techniques. Nevertheless, regulated cellular uptake of foreign materials differs according to the cell type; phagocytosis is mainly restricted to specialized cells such as macrophages, whereas pinocytosis can occur by different mechanisms in other cells by for instance clathrin- or caveolae-mediated endocytosis (Conner and Schmid 2003).

In vivo experiments have shown that alveolar macrophages contained MWCNTs after intratracheal (i.t.) instillation, pharyngeal aspiration and inhalation (Aiso et al. 2010; Elgrabli et al. 2008; Konduru et al. 2009; Mangum et al. 2006; Mercer et al. 2013a; Morimoto et al. 2012; Ronzani et al. 2012; Ryman-Rasmussen et al. 2009b). The presence of MWCNT-laden macrophages throughout the lung parenchyma in mice was noted at 30 days post-exposure following a single oropharyngeal aspiration (Wang et al. 2011). Three different types of MWCNTs, characterized as being "short" (length = 1–10 μm), "long and tangled" (length = 10–50 μm) and "long and needle-like (length = 13 μm)," were taken up by monocyte-derived macrophages from peripheral human blood (Palomaki et al. 2011). The exposure to "short and tangled" MWCNTs was shown to be associated with increased uptake in macrophages, whereas "long" MWCNTs (samples with mean length of 13 or 36 μm) resulted in incomplete uptake and frustrated phagocytosis with fibers protruding from the cell membrane or single fibers being engulfed by more than one cell (Murphy et al. 2012). In addition, MWCNTs were present in rat alveolar macrophages (RAW 264.7) after 3 h (Nagai et al. 2011) and 24 h exposure (Lee et al. 2012; Liu et al. 2012). In vitro experiments have shown that exposure to SWCNT (100 μg/ml for 24 h) in rat alveolar macrophages (NR8383) resulted in the presence of large bundles of SWCNTs in the cytoplasm (Pulskamp et al. 2007). It has been demonstrated that THP1-derived macrophages were capable of SWCNTs uptake (50 μg/ml) after 24 h exposure (Chou et al. 2008). Similarly, it

was shown by confocal microscopy that 94 % of RAW 264.7 cells had taken up fluorescent dye-coupled SWCNTs after 24-h exposure (Kosuge et al. 2012). However, other researchers have noted that RAW 264.7 cells did not actively engulf SWCNTs (Kisin et al. 2011; Shvedova et al. 2005), whereas phosphatidylserine-coated SWCNTs were efficiently engulfed (Konduru et al. 2009). This indicates that coating, and possibly also development of a lipid or protein corona, could play an important role in the uptake of CNTs by phagocytosis, which is an active process of engulfing foreign material involving recognition and actin cytoskeleton re-arrangements.

Non-phagocytic cells can take up foreign material actively by internalization. It has been shown that human microvascular endothelial cells internalized MWCNTs (length = 3.9 μm), which were subsequently located in the cytoplasm after 1 h and persisted through the 24-h exposure period (Pacurari et al. 2012). The exposure of human umbilical vein endothelial cells (HUVECs) to MWCNTs (20 μg/ml for 24 h) documented presence of fibers in vacuoles in the cytoplasm of the cells (Guo et al. 2011). Similarly, 59 and 84 % of human epidermal keratinocytes contained MWCNTs (fiber length up to 3.6 μm), localized mainly in cytoplasmic vacuoles following 24- and 48-h incubation (Monteiro-Riviere et al. 2005). Both human bronchial epithelial (BEAS-2B) and human mesothelial (MeT-5A) cells internalized CNTs during a 48-h incubation period, with CNTs located in lysozyme-like organelles or as fiber bundles in the cytoplasm (Lindberg et al. 2013). Another study also showed that human mesothelial cells internalized MWCNTs during a 3-h exposure period, whereas kidney epithelial cells did not (Nagai et al. 2011). BEAS-2B and lymphoblastoid (MCL-5) cells internalized SWCNT to the same degree regardless of the fiber length (0.4–0.8, 1–3 or 5–30 μm length), and the material was localized in membrane-bound vesicles in the cytoplasm (Manshian et al. 2013). In addition, leukemia T cells (Jurkat) internalized MWCNTs (30 μg/ml) during a 72-h exposure period (Thurnherr et al. 2009). The exposure of human type II pneumocytes (A549) and rat kidney proximal (NRK-52E) cells to MWCNT (10 μg/ml for 48 h) resulted in accumulation of materials in the cytoplasm, localized either in vesicles or as free fibers (Barillet et al. 2010; Simon-Deckers et al. 2008). The exposure to MWCNT (0.5–50 μg/ml for various periods up to 72 h) revealed materials in small vesicles in the cytoplasm of A549 cells (Foldbjerg et al. 2014; Srivastava et al. 2011). However, no internalization of SWCNTs was observed in A549 cells after a 24-h exposure period, although it should be noted that the concentrations (400 or 800 mg/ml) were rather high and TEM images of the samples indicated substantial aggregation of the fibers (Davoren et al. 2007). It is therefore possible that the dispersion of the samples had not been optimal, and single SWCNTs as well as small agglomerates cannot be visualized by traditional TEM. Still, another study also showed no internalization of MWCNTs in A549 cells after 24-h exposure to 100 μg/ml (Tabet et al. 2009). Furthermore, uptake of CNTs has also been observed in monocytes, dendritic and breast cancer cells (Foldbjerg et al. 2014; Liu et al. 2012; Müller et al. 2010).

In vitro, it has been described that CNTs were present in mesothelial cells and lymphocytes because of piercing, described as an energy-independent penetration of fibrous materials through cell membranes (Hu et al. 2010; Nagai et al. 2011). In vivo observations also show that MWCNTs (3.9 μm in length) penetrated epithelial type 1 cells and macrophages after aspiration (Mercer et al. 2010). The mechanism leading to piercing of cell membranes is poorly elucidated. However, it is not believed to be frustrated phagocytosis because it does occur in non-phagocytic cells, although there is similarity to the futile attempt to take up fibers that are longer than the cell.

Collectively, there is a substantial evidence that CNTs can be internalized by cells either by active processes (e.g., phagocytosis in macrophages) or passively by piercing. It is therefore possible that CNTs will be present in the cytoplasm as either free material or in membrane enclosures (e.g., vacuoles).

CNT exposure and oxidative stress

Oxidative stress is defined as a situation where the redox balance is shifted toward a pro-oxidant state as compared to an antioxidant state. This may be caused by increased production of oxidant species or decreased levels of free radical scavengers (e.g., ascorbate or glutathione (GSH)) or antioxidant enzymes [e.g., catalase, superoxide dismutase (SOD) or GSH peroxidase]. Oxidative stress endpoints can be assessed in experimental models ranging from simple acellular conditions to molecular epidemiology using biomarkers of oxidatively generated biomolecules, antioxidant depletion or other indicators. It is impossible to use the same assays for detection of oxidative stress in all experimental models, but they can be assembled to provide a continuum of effects from simple and well-controlled models (acellular conditions) to complex (molecular epidemiology) exposure experiments with concurrent co-exposures and effect modifiers (Fig. 2). Reactive oxygen species (ROS) production, derived from inflammation reactions, or CNTs directly, may not pose a hazard to cells unless it is associated with oxidation of biomolecules, including lipids, proteins and DNA. Oxidation products of biomolecules are used as biomarker of exposure or early biological effect in human studies on particulate matter (Møller and Loft 2010).

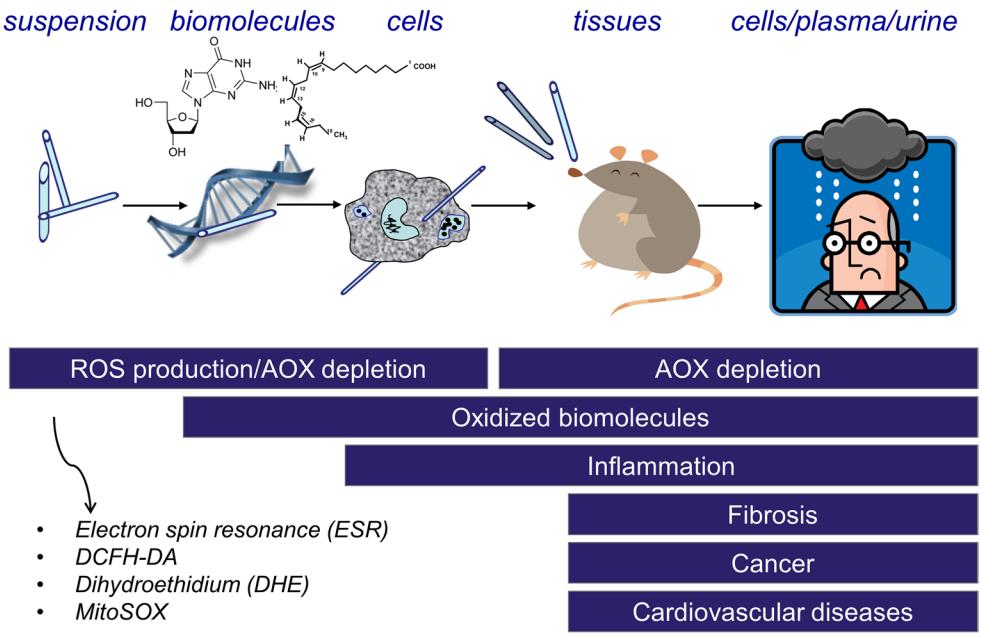

Fig. 2 The relationship between oxidative stress, inflammation and disease endpoints in experimental models. The production of reactive oxygen species can be measured both in acellular and cellular conditions, whereas oxidatively generated biomolecules are more often used in animals and humans as indicators of oxidative stress

Measurement of ROS production

Free radicals can be measured by electron spin resonance (ESR), typically with a spin trap agent such as 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). This technique is used for assessment of hydroxyl radical production by transition metals in acellular conditions with hydrogen peroxide. In cell culture experiments, it is common to use molecular probes for ROS production. These probes are not particularly specific for a given type of ROS. However, the dihydroethidium (DHE) assay is typically regarded as a quantification tool for superoxide anion radicals, used especially in cells with phagocytosis activity because respiratory burst generates superoxide anion radicals among several oxidizing species. Dichlorodihydrofluorescein diacetate (DCFH-DA) has been very popular as probe for ROS production in nanotoxicology, measuring a range of oxidizing species such as hydroxyl radicals and peroxynitrite (Stone et al. 2009). Other assays such as Amplex Red and MitoSOX seem to have been used less frequently in studies of ROS production by CNTs (Ju et al. 2014; van Berlo et al. 2014).

ROS production by CNTs in acellular conditions

Certain studies using DCFH have demonstrated ROS production by SWCNTs and MWCNTs in acellular conditions (Cao et al. 2014; Folkmann et al. 2009; Jacobsen et al. 2008; Thurnherr et al. 2009; Vesterdal et al. 2014b). SWCNTs with high iron content (26 %) generated radical signals in ESR, whereas SWCNTs with low content of iron (0.23 %) showed no detectable signals (Kagan et al. 2006; Shvedova et al. 2005). Other studies utilizing ESR (with or without DMPO as spin trap) showed no ROS production or carbon-centered radicals, whereas MWCNTs even scavenged free radicals (Fenoglio et al. 2006; Porter et al. 2013). Five different types of MWCNTs, described as "long needle-like" (2 types), "long entangled," "short purified" and "short non-purified" also scavenged ROS in suspension with hydrogen peroxide, assessed by ESR with DMPO as spin trap agent (Nymark et al. 2014). Double-walled CNTs with hydrogen peroxide also abolished free radical signal in ESR, using α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone as the spin trap agent (Crouzier et al. 2010). In addition, the exposure to MWCNTs was not associated with increased ROS production, as determined by the DHE assay (Pacurari et al. 2012) or oxidation product of 2,2-diphenyl-1-picrylhydrazyl probe (Ma-Hock et al. 2009). In general, it seems that the ability of CNTs to generate ROS in acellular conditions depends on the type of material, impurities and assay.

ROS production by CNTs in cell cultures

Investigations of ROS production in cell cultures have either measured this directly during the incubation with CNTs or have measured the ROS production after an incubation period with CNTs. Early observations showed that SWCNTs with high residual iron content (30 % by mass) increased the intracellular ROS production, assessed by ESR (Shvedova et al. 2003). The ROS production was attributed to the high content of iron, which was supported by observations that addition of catalase or deferoxamine (DFO) iron chelator abolished the ROS production. Later studies also indicated increased ROS production by ESR,

DHE or DCFH in mesothelial cells by exposure to SWCNTs with low iron content (0.07 % by weight), which was attenuated by pre-treatment with catalase, SOD or DFO (Pacurari et al. 2008). ROS production assessed by the Amplex Red assay was also increased during the incubation period with two different types of MWCNTs in RAW 264.7 cells (van Berlo et al. 2014). Other studies showed increased ROS production of SWCNTs by the DCFH assay in keratinocytes (HaCaT) (Manna et al. 2005), FE1-MML lung epithelial cells (Jacobsen et al. 2008), HUVECs (Vesterdal et al. 2014b), A549 cells (Thurnherr et al. 2011) and primary mouse embryo fibroblasts (Yang et al. 2008a). SWCNT-mediated ROS production in human foreskin cells was attenuated by treatment with GSH and *N*-acetylcysteine (NAC), a thiol compound with antioxidant capacity (Sarkar et al. 2007). A direct comparison of different types of SWCNT showed that especially materials with 1–3 μm fiber length were associated with increased intracellular ROS production, whereas SWCNTs with shorter (0.4–0.8 μm) or longer (5–30 μm) fiber length had less ROS production, assessed by the DCFH assay (Manshian et al. 2013). The same pattern was observed in Jurkat cells where MWCNTs from three different suppliers with 2–4 μm fiber length had more intracellular ROS production (DCFH assay) as compared to samples with 2 and 2–16 μm fiber length (Thurnherr et al. 2009). However, there is also a study showing no ROS production in BEAS-2B cells after exposure to MWCNTs (Tsukahara et al. 2013). Using a relatively short exposure time (30 min) and high concentrations (80–640 μg/ml, corresponding to 10–80 μg/cm^2), it was shown that long needle-like MWCNTs scavenged ROS in BEAS-2B, assessed by ESR with DMPO (Nymark et al. 2014). There was no ROS production in RAW 264.7 cells after exposure to SWCNT with low iron content, assessed by DHE assay (Shvedova et al. 2005). The same authors also demonstrated that SWCNTs with different content of iron (0.23 and 26 %) displayed no intracellular ROS production in RAW 264.7 cells by measurement with the ESR and DHE assays (Kagan et al. 2006; Kisin et al. 2011).

Certain studies have shown that pre-incubation of CNTs in macrophages (RAW 264.7 or NR8383), primary aortic endothelial cells, human gingival fibroblasts, rat kidney proximal (NRK-52E), hepatocytes (HepG2), lung fibroblasts, BEAS-2B, A549, breast cancer cells (MCF-7) and neuroblastoma cells primed these to generate ROS after removal of CNTs as detected by the DCFH assay (Alarifi et al. 2014; Barillet et al. 2010; Chen et al. 2011; Cheng et al. 2012; Cicchetti et al. 2011; Di Giorgio et al. 2011; Guo et al. 2011; He et al. 2011; Liu et al. 2012; Schrand et al. 2007). Another study showed that SWCNTs primed murine macrophages (J774) to ROS production, whereas A549 and THP1 cells did not increase the ROS production (Foldbjerg et al. 2014). The ROS production in MWCNT-exposed A549 cells was not decreased by inhibition of mitochondrial activity (rotenone), suggesting other sources of intracellular ROS production than the electron transport chain in CNT-stimulated cells (Srivastava et al. 2011). This is in accordance with observations that A549 cells have little mitochondrial function (Jantzen et al. 2012). Nevertheless, the priming of ROS production after 24-h exposure in A549 cells to either SWCMTs or MWCNTs was attenuated by pre-treatment with NAC (Pulskamp et al. 2007). Similarly, a 24-h incubation period with MWCNTs increased the ROS production in A549 cells, which was attenuated by addition of GSH or NAC (Ye et al. 2009). Another study showed that A549 cells had increased ROS production (measured with DCFH or MitoSOX probes) following 2- to 24-h exposure to MWCNTs, which was blunted by treatment with NAC (Ju et al. 2014). It has also been shown that exposure to MWCNTs was associated with increased ROS production in human microvascular epithelial cells, which was attenuated by pre-treatment with catalase (Pacurari et al. 2012). MWCNTs increased ROS production by the DCFH assay in human hepatoblastoma C3A cells, which was inhibited by pre-treatment with Trolox—a vitamin E analogue (Kermanizadeh et al. 2012). The exposure of the same types of MWCNTs to human renal cells did not increase the ROS production, assessed by oxidation of DHE as probe for superoxide anion radicals (Kermanizadeh et al. 2013). Unaltered ROS production was also observed in human neuroblastoma (SH-SY5Y) cells after 72-h exposure to functionalized MWCNTs (5 μg/ml), which might have been due to insufficient time of the post-exposure incubation with DCFH (Vittorio et al. 2009). Another study showed that pre-exposure to MWCNTs did not affect the ROS production potential in rat pheochromocytoma (PC12) cells during a 1-h incubation period with DCFH (Xu et al. 2009). It was also reported that A549 cells, monocytes, dendritic cells or a triple culture of these cells did not increase ROS production and there was unaltered total antioxidant capacity (measured by a commercial kit) after a 24-h exposure period to SWCNTs (Müller et al. 2010).

Differences between studies showing increased ROS production or no effect can be due to a number of factors, including the type of CNT material, cell type and exposure scenarios. The dispersion protocol differences have attracted much attention as the source of variable results between the different studies. However, it should be emphasized that even poorly dispersed samples of CNTs can be associated with increased intracellular ROS production. It has been shown that the ROS production in bronchial (NHBE) or A549 cells was dependent on the vehicle for dispersion of SWCNTs, where addition of dipalmitoyl phosphatidyl choline (a lung surfactant) and fetal bovine serum increased and decreased the ROS production,

respectively (Herzog et al. 2009). Another indirect way causing elevated levels of intracellular ROS is depletion of nutrients in the cell culture medium. This has been documented to affect the cytotoxicity of A549 cells (Casey et al. 2008). Nevertheless, formation and release of ROS and myeloperoxidase (MPO) products from cells could be important for slow elimination of CNT where phagocytosis fails (Kagan et al. 2014; Kotchey et al. 2013).

ROS production by respiratory burst in CNT-exposed cells

Respiratory burst during phagocytosis in professional phagocytes (neutrophils and macrophages) is mediated by NADPH oxidase, which assembles in the plasma membrane or phagosomes. The activation of NADPH oxidase, by dual action with MPO, generates ROS that are instrumental in the phagocyte microbial function (Nordenfelt and Tapper 2011; Nunes et al. 2013). However, frustrated phagocytosis occurs when phagocytes attempts to engulf materials that are longer than the diameter of the cell (typically 12–20 μm in length). This process has mainly been demonstrated in macrophages with respect to exposure to fibers, which could be due to the fact that phagocytosis is much easier to study in macrophages as compared to neutrophils (Nordenfelt and Tapper 2011; Nunes et al. 2013). The consequence of frustrated phagocytosis is a prolonged ROS production and secretion of cytokines that may damage adjacent cells (Murphy et al. 2012; Schinwald and Donaldson 2012). Increased ROS production, associated with frustrated phagocytosis, has been observed in cultured monocytic cells following exposure to CNTs (Brown et al. 2007). Interestingly, it has been shown that macrophages from rat alveolar macrophages had higher phagocytic activity, and hence also higher degree of frustrated phagocytosis, than alveolar macrophages following exposure to man-made synthetic vitreous fibers (Dörger et al. 2001). There may also be species differences as demonstrated in a study showing that rat alveolar macrophages had higher degree of frustrated phagocytosis following exposure to man-made synthetic vitreous fibers than alveolar macrophages from hamsters (Dörger et al. 2000). By analogy, similar differences may be observed in cells following exposure to CNTs.

CNT-mediated ROS production in animals

There are very few studies on ROS production in vivo after exposure to CNTs. However, one study exposed mice by intra-nasal instillation of double-walled CNTs (1.5 mg/kg) and measured the ROS production in an organic extract of the lung tissue at 1 h after administration of a spin trap for ESR measurements (Crouzier et al. 2010). Somewhat surprisingly there was decreased ROS production in lung tissue at 24 and 48 post-exposure, which the authors attributed to a scavenger effect of the CNTs (Crouzier et al. 2010). However, it should be noted that the exposure route (intra-nasal instillation) was not optimal physiologically and the ex vivo measurement of ROS production in a lipid-soluble extract of tissue may not be representative of the toxicological mechanism in the lung following inhalation of CNTs.

Effect of CNT exposure on antioxidant level in cells

A number of studies have investigated the effect on antioxidants or enzymes by CNT exposure in cells. A general issue regarding these observations is that effects in either direction can be interpreted as sign of oxidative stress. Reduced levels of endogenous antioxidants indicate oxidative stress, whereas increased levels of the same antioxidant can be interpreted as a response to oxidative stress. For instance, it was shown that 24-h exposure to functionalized SWCNT in Caco-2 cells increased the activity of catalase and SOD (presumably due to increased protection against oxidative stress) and depleted GSH levels (Pichardo et al. 2012). Certain studies have shown decreased content of GSH and SOD activity in cultured RAW 264.7, murine alveolar macrophages and A549 cells after exposure to MWCNTs (Aldieri et al. 2013; Chen et al. 2011; Han et al. 2012; Reddy et al. 2011). GSH depletion was observed in RAW 264.7 cells after exposure to SWCNTs with different content of iron (0.23 and 26 %), whereas there were no signs of intracellular ROS production by ESR and DHE assays (Kagan et al. 2006; Kisin et al. 2011). Incubation of human epidermal keratinocytes (HaCaT) with SWCNTs for 18 h decreased the intracellular content of antioxidants (GSH, α-tocopherol) and increased intracellular ROS levels (Shvedova et al. 2003). However, SWCNTs exposure increased both ROS production and total GSH content in human colon carcinoma HT29 cells (Pelka et al. 2013). It was shown that MWCNTs exposure depleted intracellular GSH content, concurrently with increased ROS production by the DCFH assay in human hepatoblastoma C3A cells (Kermanizadeh et al. 2012). These studies on mono-cultures of cells do not take into account that tissues contain different types of cells, each having special functions. Recent studies have used more advanced models that mimic the functions or architecture of tissues. Monocultures of human monocyte-derived macrophages, dendritic or lung epithelial cells, and the triple co-culture of these cells, had reduced GSH levels after exposure to CNTs (Clift et al. 2014). Using rat lung slices, it was shown that one out of three tested MWCNTs slightly increased the GSH content, which was attributed to increased protection against oxidative stress (Sauer et al. 2014).

Effect of CNT exposure on antioxidant levels in animals

In vivo studies have shown GSH depletion and reduced levels of SOD and catalase activity after exposure to relatively high doses of aerosolized CNTs by nose-only inhalation in mice (5 mg/kg per day delivered for 20 min/day for 7 consecutive days) (Ravichandran et al. 2010). Oropharyngeal aspiration of MWCNT (20 or 40 μg/mouse) was associated with decreased extracellular levels of SOD in lung tissue, whereas there were unaltered levels of SOD in serum (Han et al. 2010). MWCNTs (20 μg/mouse by pharyngeal aspiration), administered with or without ozone inhalation (0.5 ppm for 3 h), did not increase the level of SOD in lung tissue (Han et al. 2008).

Slightly reduced GSH and SOD activity levels in the liver were observed in mice at day 15 after intravenous (i.v.) injection of 60 mg/kg of functionalized MWCNTs, which also displayed signs of hepatic injury in terms of increased plasma concentrations of aspartate aminotransferase (Ji et al. 2009). Intravenous injection of SWCNTs was associated with reduced levels of GSH in liver and lungs, but not the spleen at 90 days post-exposure, whereas the same doses were not associated with changes in serum TNF levels (Yang et al. 2008b).

Effect of CNT exposure on heme oxygenase 1 levels

The expression of *heme oxygenase (decycling) 1* (*Hmox-1*) or the protein level has been used as a general indicator of oxidative stress. This gene contains the antioxidant response element, which is located in the promotor region of certain genes that encode for proteins in the antioxidant defense system (Reddy et al. 2011). The redox-regulated transcription factor Nrf2 increases the gene expression of *Hmox-1* through binding to this element (Ma 2013). Hmox-1 confers cytoprotective effects by catalyzing the degradation of toxic-free heme to less hazardous degradation products (Gozzelino et al. 2010). It has been shown that the Hmox-1 protein levels were increased in aortic endothelial cells 72–96 h post-exposure to SWCNTs (Cheng et al. 2012). Gene array analysis of human BJ foreskin cells indicated that *Hmox-1* was upregulated after 24-h exposure to SWCNTs (Sarkar et al. 2007).

A repeated exposure study of MWCNTs (25.6 μg/week for 5 weeks by i.t. instillation) in mice showed increased levels of *Hmox-1* in lung tissue at 24 h after the last exposure, whereas there were unaltered levels in the liver (Cao et al. 2014). Interestingly, it was observed that the expression of *Hmox-1* was increased in lung, aorta and heart tissue at day 7 after pharyngeal aspiration of SWCNTs in mice, whereas there were unaltered levels at post-exposure day 1 and 28 (Li et al. 2007b). Another study showed increased *Hmox-1* gene expression in aorta tissue at 4 h post-exposure to pharyngeal aspiration (40 μg) in mice (Erdely et al. 2009). However, there are also observations of unaltered *Hmox-1* expression levels in lung tissue at 2 h after the last of two exposures (total dose = 1 mg/kg) by i.t. instillation in mice (Vesterdal et al. 2014b). This is similar to observations of unaltered Hmox-1 levels in lung tissue, measured by ELISA, at day 3, 30 or 90 following inhalation of SWCNTs (0.03 mg/m^3, 6 h/day, 5 days/week) for 4 weeks in rats (Morimoto et al. 2012). Unaltered expression levels of *Hmox-1* were observed in lung tissue at 8 weeks after pulmonary exposure to MWCNTs (2 mg/kg) in mice by pharyngeal aspiration (van Berlo et al. 2014). Mice had increased expression of *Hmox-1* in heart tissue and mitochondrial oxidative stress (reduced GSH and increased level of protein carbonyl groups) after the exposure to SWCNTs (Li et al. 2007b). Gastrointestinal exposure of rats to SWCNTs also did not change the *Hmox-1* expression in liver and lung tissue at 24 h post-exposure (Folkmann et al. 2009).

Collectively, several lines of evidence indicate that exposure to CNTs is associated with oxidative stress in cell cultures and animals. Increased intracellular ROS production has been documented in several studies on different cell types. However, the data are conflicting with regard to the CNTs ability to produce ROS. Cellular sources of ROS production may be more important than direct ROS production by CNTs. There is also evidence that exposure to CNTs is associated with depletion of antioxidants and replenishment of these is associated with blunted cellular ROS production. One consequence of this elevated level of ROS production is cell signaling to increase the antioxidant defense system (e.g., increased *Hmox-1* expression).

CNT exposure and inflammation responses

The effect of CNT exposure on inflammation has been investigated in several cell types and animal models, usually related to pro-inflammatory signaling, secretion of cytokines or secondary events such as increased expression of intercellular vascular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), selectin E (SELE) or selectin P (SELP). It has been shown that SWCNT exposure promoted activation of nuclear factor of kappa B (NF-κB) in HaCaT and mesothelial cells, with subsequent activation of mitogen-activated protein (MAP) kinases (Lee et al. 2012; Manna et al. 2005; Pacurari et al. 2008). Similar observations were obtained after SWCNT exposure to THP1-derived macrophages, which led to activation of NF-κB and activator protein 1 (AP-1) (Chou et al. 2008). The activation of NF-κB functions as a master switch leading to pro-inflammatory signaling and secretion of cytokines. Another important pathway for inflammatory

signaling is activation of NOD-like receptor pyrin domain containing three (NLRP3) inflammasome, leading to maturation of IL1β. This pathway is implicated in the sensing of hazardous materials such as asbestos and silica, triggered by ROS production from NADPH oxidase (Hornung et al. 2008; Wang et al. 1999). Inflammasome activation was observed in monocyte-derived macrophages from peripheral blood after exposure to long, needle-like MWCNTs, whereas short and long, tangled fibers did not promote this activation (Palomaki et al. 2011). The involvement of ROS production was documented by reduced IL1β secretion in cells that were also exposed to NAC or an NADPH oxidase inhibitor (Palomaki et al. 2011). However, the inflammasome activation depends also on other factors such as cathepsin B activity, P2X7 receptor activation, SRC and TYR tyrosine and Rho kinases (Kanno et al. 2014). The complexity of the inflammation signaling in CNT-exposed cells is documented by the wide array of secreted cytokines, differing between varying cell types. For instance, it has been shown that exposures to MWCNTs in primary peritoneal or monocyte-derived macrophages were associated with increased TNF production (Clift et al. 2014; Gasser et al. 2012; He et al. 2011; Liu et al. 2012; Muller et al. 2005), whereas exposure to CNTs did not increase the TNF secretion in RAW 264.7 cells (Di Giorgio et al. 2011). However, secretions of other inflammatory proteins (IL1β, IL6, IL10, CCL2 and prostaglandin E2) from CNT-exposed RAW 264.7 cells have been documented (He et al. 2011; Lee et al. 2012). CNTs did not increase the release of IL8 from A549 cells, or TNF from rat alveolar (NR8383) macrophages after 24-h exposure (Pulskamp et al. 2007). It was also reported that A549 cells, monocytes, dendritic cells or a triple culture of these cells mainly had unaltered IL8 and TNF secretion after a 24-h exposure period to SWCNTs (Müller et al. 2010). Using a panel of MWCNTs with different structures, it was shown that "long" fibers (two different samples with average length of 13 and 36 μm) increased the cytokine secretion of IL1β, IL6 and IL8 (but not TNF) in THP1-derived macrophages, whereas mesothelial cells did not produce any cytokines unless they were exposed to conditioned medium from MWCNT-exposed macrophages (Murphy et al. 2012).

Inflammation signaling also occurs in cells that are not primarily involved in the immune system. NF-κB activation and increased *TNF* expression have been observed in primary aortic endothelial cells after exposure to SWCNTs (Cheng et al. 2012). The incubation of human aortic endothelial cells with MWCNTs, suspended in surfactant, was associated with more pronounced gene expression of pro-inflammatory cytokines (*IL8*, *CCL2*), cell adhesion molecule expression (*VCAM1*, *SELE*) and CCL2 secretion as compared to MWCNTs suspended in cell culture medium (Vidanapathirana et al. 2012). Similar observations were obtained by exposure of human microvascular endothelial cells to MWCNTs, which increased the secretion of CCL2 and ICAM-1 (Pacurari et al. 2012). There are also observations showing that exposure to MWCNTs was associated with increased secretion of IL8 from HEK293 (Monteiro-Riviere et al. 2005) and epidermal cells (Reddy et al. 2011). The exposure to MWCNT increased IL8 secretion in A549 cells, which was reduced by treatment with GSH or NAC (Ye et al. 2009). Another study showed that pre-treatment with Trolox reduced MWCNT-induced IL8 production in human hepatoblastoma cells (Kermanizadeh et al. 2012). The same types of MWCNTs also increased the levels of IL6 and IL8 in renal cells, whereas CCL2 and TNF levels were unaltered (Kermanizadeh et al. 2013).

Inflammation in the lungs after airway exposure to CNTs is typically assessed by influx of polymorphonuclear neutrophils (PMNs) or other immune cells in bronchoalveolar lavage fluid (BALF), level/expression of pro-inflammatory mediators or histology. The extent of pulmonary inflammation depends on both the dose and dose rate (Oberdörster 2002). In particular, i.t. instillation (or pharyngeal aspiration) may mount very strong pulmonary inflammation associated with influx of PMNs in BALF, increased levels of cytokines or MPO activity (Aiso et al. 2010; Cao et al. 2014; Erdely et al. 2009; Ge et al. 2012; Han et al. 2008, 2010; Jacobsen et al. 2009; Muller et al. 2005; Murray et al. 2012; Nemmar et al. 2007; Shvedova et al. 2005, 2008b; Urankar et al. 2012; Wang et al. 2011). Moreover, one study showed that pre-treatment with α-tocopherol blunted the SWCNT-mediated pulmonary inflammation in terms of lower levels of PMNs, IL6 and TNF in BALF (Shvedova et al. 2007). However, a few studies have not observed pulmonary inflammation at 24 h after a single i.t. instillation to MWCNTs at doses up to 100 μg/rat (Elgrabli et al. 2008; Thompson et al. 2014). This is in keeping with observations that i.t. instillation at 160 μg/rats produced a marked pulmonary inflammation, whereas a dose of 40 μg/ml was only associated with moderate infiltration of macrophages and unaltered levels of neutrophils (Aiso et al. 2010). Other studies on i.t. instillation of CNTs in rats have also used higher doses (600 or 2,000 μg/rat as lowest dose), showing pulmonary inflammation (Ge et al. 2012; Muller et al. 2005). The null-effect studies therefore may have used doses that were too low to mount an inflammatory response following i.t. instillation. Likewise, a number of studies on inhalation exposure have documented influx of PMNs in BALF (Porter et al. 2013; Ravichandran et al. 2010; Ryman-Rasmussen et al. 2009b; Shvedova et al. 2008a; Stapleton et al. 2012) or no pulmonary inflammation (Kim et al. 2014; Mitchell et al. 2007) in rats or

mice after exposure to CNTs. In addition, the fiber length/dimension is important for the pulmonary inflammation response (Donaldson et al. 2010), as well as the surface area/reactivity and lung burden of fibers (Pauluhn2014). Early investigations demonstrated that i.t. instillation of SWCNTs at a dose of 5 mg/kg (but not 1 mg/kg) in rats was associated with increased influx of PMNs in BALF (24 h post-exposure) and development of multifocal granulomas at later time points (Warheit et al. 2004). It has also been shown that i.t. instillation of three different types of SWCNTs in mice (0.1 or 0.5 mg/mouse) caused pulmonary inflammation and formation of granulomas, regardless of the material having high content of iron (27 % by weight), nickel (26 %) or low concentration of metal impurities (Lam et al. 2004). However, a study comparing different lengths of MWCNT administered by intratracheal spraying every 2 weeks for 24 weeks in rats showed that the long type induced more pleural inflammation, whereas the short type induced more pulmonary tissue inflammation consistent with the translocation pattern (Xu et al. 2014).

Signs of systemic inflammation and oxidative stress may occur fast after pulmonary exposure as documented by increased gene expression of cytokines, *Hmox-1* and *Sele* in aortic tissue at 4 h following pharyngeal aspiration of 40 µg/mouse of CNTs (Erdely et al. 2009). The exposure to CNTs by pharyngeal aspiration (40 µg/mouse) also increased serum and liver levels of acute phase proteins (C-reactive protein, haptoglobin and serum amyloid A) and promoted elevated numbers of neutrophils in blood of MWCNT-exposed mice (Erdely et al. 2011). This is supported by a 90-day inhalation study, showing blood neutrophilia in rats after exposure to 2.5 mg/m^3 of MWCNTs (6 h/day and 5 days/week), whereas concentrations of 0.1 or 0.5 mg/m^3 displayed no signs of systemic inflammation as judged by OECD guideline test number 413 (Ma-Hock et al. 2009). Another study exposed rats to 0.1–6 mg/m^3 of MWCNT (6 h/day, 5 days/week for 13 weeks) and observed no systemic changes related to the endpoints outlined by the OECD guideline test number 413 (Pauluhn 2010). Still, inhalation of 1 or 5 mg/m^3 of MWCNT for 14 days (6 h/day) was associated with systemic immunosuppression and increased the expression of *NAD(P)H oxidoreductase 1* (an antioxidant gene) and *IL10* (and IL10 protein) in the spleen, whereas *IL6* expression levels were unaltered (Mitchell et al. 2007).

Collectively, there is evidence from cell cultures and animals that exposure to CNTs is associated with pro-inflammatory signaling in cells that are involved in the immune system and target tissue cells of epithelial and endothelial origin. The type of CNT and dose are important determinants for inflammatory signaling.

DNA damage in cells and tissue after pulmonary or non-pulmonary exposure to CNTs

Oxidative stress-generated DNA lesions are typically segregated in small nucleobase lesions or larger DNA adducts (Loft et al. 2008). The former encompasses small oxidatively damaged DNA lesions such as 8-oxo-7,8-dihydro-guanine (8-oxoGua), which is most commonly measured as the 2′-deoxynucleoside 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (Cooke et al. 2010). This DNA lesion is mutagenic, giving rise predominantly to G to T transversions in mammalian cells (Kamiya et al. 1992; Le Page et al. 1995; Moriya 1993; Tan et al. 1999). In addition, 8-oxodG is frequently found in lung cancer tissue (Møller et al. 2010). However, cellular DNA typically contains at least 11 other oxidatively generated nucleobase lesions, encompassing 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde), uracil glycol, thymine glycol, 5-hydroxycytosine and 5-hydroxyuracil (Cadet et al. 2012). The mechanism of mutagenicity of formamidopyrimidine lesions is unraveled in mammalian cells, although it is now clear that the lesions give rise to G to T transversion mutations (Gehrke et al. 2013). The small nucleobase lesions can be measured by chromatographic methods coupled to either electrochemical detection or tandem mass spectroscopy. In this respect, it should be noted that certain protocols for DNA processing is associated with spurious oxidation, which increases the baseline level of DNA oxidation products and seriously distorts the measurements. It has been recommended by the European Committee on Oxidative DNA Damage (ESCODD) that any investigation with baseline levels of 8-oxodG in control groups above 5 lesions/10^6 dG should be interpreted with skepticism (ESCODD 2003a, b). This issue concerns certain articles on genotoxicity of CNTs (Kato et al. 2013; Ogasawara et al. 2012). As 8-oxodG is considered to be the most predominant small oxidatively generated DNA base lesion, this ESCODD threshold of flawed results should be applicable to other lesions as well. In addition, 8-oxodG is often measured by antibody-based methods (immunohistochemistry or ELISA), but these assays yield higher levels of lesions than the chromatographic assays, possibly because of non-specific binding to other biomolecules in cells or fluids by the antibodies (Barregard et al. 2013). Certain studies on CNT-generated 8-oxodG have used antibody-based methods (Inoue et al. 2010; Mohiuddin et al. 2014). This review will adhere to the critical assessment of the analysis of the studies with poor assays utilized (antibody-based or chromatographic assays with high baseline levels of 8-oxodG or other small oxidatively damaged DNA lesions are mentioned, but will not give any weight to the overall assessment of the association between CNT exposure and effect).

A different option for the measurement of oxidatively damaged DNA is using repair enzymes that cleave the DNA

strand at sites of oxidized lesions. In particular, the alkaline comet assay has been popular in particle genotoxicity, in which oxidatively damaged DNA lesions are measured by incubation of DNA with formamidopyrimidine DNA glycosylase (FPG), endonuclease III (ENDOIII) or oxoguanine DNA glycosylase (hOGG1). The hOGG1 enzyme is considered to detect 8-oxoGua, whereas the FPG enzyme also detects ring-opened formamidopyrimidine lesions (FapyGua and FapyAde). ENDOIII lesions encompass oxidized pyrimidines such as uracil glycol, thymine glycol, 5-hydroxycytosine and 5-hydroxyuracil (Evans et al. 2004). The alkaline comet assay also detects strand breaks (SB), which can be generated by oxidizing agents such as X-rays in lung tissue after topical irradiation on thorax (Risom et al. 2003). X-rays are also used for calibration of the comet assay because there is a well-known linear relationship between the exposure to ionizing radiation and SB generation (Møller et al. 2012). However, it should be noted that exposure to certain non-oxidizing agents (for instance heterocyclic aromatic amines) can give rise to SB because the primary DNA lesions are labile in alkaline solution or detection of transient breaks in DNA due to repair processes (Møller 2005). Therefore, the measurement of SB in cells has relevance as a genotoxic endpoint, although it may not necessarily be a marker of oxidative stress. This also means that the total level of SB after treatment with enzymes encompasses both oxidative stress-generated DNA nucleobase lesions and lesions that may not be generated by oxidation reactions. It is therefore common practice to subtract the basal level of SB to obtain enzyme-sensitive sites.

Another troublesome issue regarding genotoxicity studies by the alkaline comet assay is the use of all measured comets for the statistical analysis. This problem may be especially relevant in cell culture studies of genotoxicity. It is a common practice to assess the level of DNA damage in three or more independent experiments, preferably with cell cultures in different passages. In this assay, DNA migration is assessed in 50–100 comets per gel, and the mean or median is subsequently calculated from this distribution of comets, which gives rise to one value ($n = 1$). However, certain studies seem to have used all comets in the statistical analysis (i.e., $n \geq 100$), which hugely inflates the statistical power. A number of studies have not been assessed in the present review due to this issue or uncertainty about the number of independent replicates in the experimental protocol (Cheng et al. 2012; Ogasawara et al. 2012; Yamashita et al. 2010; Zeni et al. 2008).

Oxidatively generated DNA lesions in cell cultures

A number of studies have observed that exposure to CNTs increased levels of SB, measured by the alkaline comet assay in primary cultures or cell lines from bronchial epithelium (Lindberg et al. 2009), alveolar type II pneumocytes (Cavallo et al. 2012; Karlsson et al. 2008), colon carcinoma (Pelka et al. 2013), mesothelium (Pacurari et al. 2008), renal proximal tubule epithelium (Kermanizadeh et al. 2013), kidney proximal (NRK-52E) tissue (Barillet et al. 2010), hepatocytes (Alarifi et al. 2014; Kermanizadeh et al. 2012; Vesterdal et al. 2014a), lung fibroblasts (Kisin et al. 2007, 2011), embryo fibroblasts (Yang et al. 2008a), gingival fibroblasts (Cicchetti et al. 2011) and macrophages (Di Giorgio et al. 2011). However, there are also studies showing no effect on SB levels after exposure to MWCNTs in keratinocytes (McShan and Yu 2012) and SWCNTs in lung epithelial cells (Jacobsen et al. 2008; Thurnherr et al. 2011). Figure 3 outlines the magnitude of effect in different studies of CNT-generated SB. The effect size, assessed as fold-increase per concentration unit (μg/ml or μg/cm^2) as compared to the unexposed cells, differs about three-orders of magnitude between the studies. Several studies have not provided enough information about particle characteristics for assessment of determinant factors across studies. Nevertheless, the aggregated results from Fig. 3 indicate that MWCNTs and SWCNTs have similar potential to generate SB. Nevertheless, a further attempt to identify factors (i.e., fiber length, specific surface area, metal impurities of CNTs or differences in incubation periods and cell types) for the observed difference in effect has not been successful as demonstrated in supplementary table S3, which includes additional information on fiber length, specific surface area, transition metal content, incubation time and cell type. Considering the association between iron content and ROS production in cells outlined above a similar association with DNA damage might be expected although there

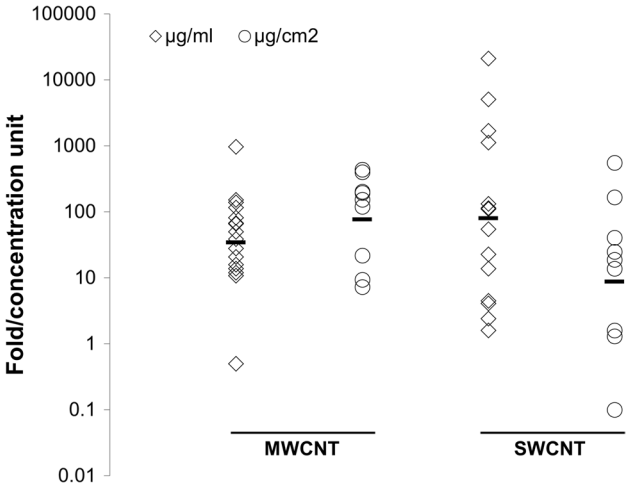

Fig. 3 The association between exposure to carbon nanotubes and induction of DNA strand breaks in cell cultures with each point representative of one study. The *vertical lines* represent the geometric means

are multiple differences with expect to exposure conditions. Moreover, antioxidants such as ascorbate and GSH can under some circumstances enhance rather than prevent oxidative damage to DNA induced by transition metals, such as iron (Fischer-Nielsen et al. 1992).

Certain studies have observed increased levels of oxidatively damaged DNA, using the enzyme-modified version of the comet assay. The exposure to SWCNT increased the level of FPG-sensitive sites in FE1-MML lung epithelial and HepG2 cells, whereas SB levels were only increased in the latter (Jacobsen et al. 2008; Vesterdal et al. 2014a). Both SWCNT and MWCNT increased the level of SB, ENDOIII- and FPG-sensitive sites in RAW 264.7 cells (Migliore et al. 2010). However, unaltered levels of FPG-sensitive sites were observed in A549 cells (Cavallo et al. 2012; Karlsson et al. 2008).

A number of studies have reported the total level of DNA damage after treatment with base excision repair enzymes. Where possible, we have subtracted the basal level of SB for the interpretation of the level of enzyme-sensitive sites. Exposure of SWCNTs to human colon carcinoma (HT29) cells was associated with increased levels of SB, whereas FPG sites were unaltered (Pelka et al. 2013). The total level of DNA lesions after FPG treatment was increased after exposure to MWCNTs in human hepatoblastoma cells, but the level of FPG-sensitive sites appears not to have been different between exposed and control cells (Kermanizadeh et al. 2012). The same authors also showed that FPG-treated samples had fewer lesions than the corresponding determination of SB by the same types of MWCNTs, indicating unaltered levels of FPG-sensitive sites (Kermanizadeh et al. 2013). The exposure to MWCNTs in HaCaT cells was associated with increased levels of FPG total sites, whereas there was no difference in levels of SB (McShan and Yu 2012).

Certain studies have investigated levels of 8-oxodG in cells or animal tissues by antibody-based techniques, showing increased level of DNA damage by MWCNTs (Mohiuddin et al. 2014) and SWCNTs (Inoue et al. 2010). One study reported increased levels of 8-oxodG by HPLC-ECD in mesothelial cells after 24-h exposure to CNTs, but with high baseline levels (7.6 lesions/10^6 dG), indicating spurious oxidation of DNA during processing of samples (Ogasawara et al. 2012).

Levels of oxidatively damaged DNA in CNT-exposed animals

Studies on oxidatively damaged DNA have mainly focused on pulmonary exposure to CNTs. Increased SB levels in BALF cells were observed at 3 h after i.t. instillation of SWCNTs (54 μg/mouse), concurrently with increased expression of *Mip-2*, *Ccl2* and *IL-6*, whereas increased levels of neutrophils were observed at 24 h after the exposure (Jacobsen et al. 2009). A single i.t. instillation of MWCNTs (50 or 200 μg/mouse) was associated with increased levels of SB (3 h post-exposure) and lipid peroxidation-derived DNA lesions (3–168 h) in lung tissue (Kato et al. 2013). However, a lower dose of SWCNTs (approximately 10 μg/mouse as total dose administered by i.t. instillation at 26 and 2 h before killing) did not increase the level of SB and FPG-sensitive sites in lung tissue, and there was unaltered expression of *Hmox-1*, *Ccl2*, *Icam-1*, *Vcam-1* and *Ogg1* (Vesterdal et al. 2014b).

A repeated exposure study with MWCNTs (25.6 μg/week for 5 weeks by i.t. instillation) showed pulmonary inflammation (PMNs and cytokines in BALF) and elevated levels of SB in lung tissue, whereas there were unaltered levels of FPG-sensitive sites (Cao et al. 2014). Another study on repeated i.t. instillations of MWCNT or SWCNT (0.2 or 1 mg/kg, or 0.04 or 0.2 once a week for 5 weeks) showed pulmonary inflammation by histopathological examination, whereas there were no differences in lung levels of SB (Ema et al. 2013; Naya et al. 2012).

A study comparing different lengths of MWCNT administered by i.t. spraying every 2 weeks for 24 weeks in rats showed that mainly the short type induced guanine oxidation in lung tissue consistent with distribution and inflammation patterns (Xu et al. 2014). However, it should be emphasized that the methodology was not reported and the levels of DNA damage in unexposed rats were rather high (1.3 ng/μg DNA, corresponding to 7600 lesions/10^6 dG).

Whole-body inhalation (0.16–0.94 mg/m^3, 6 h/day for 5 days) of MWCNTs with a length of 2.6 μm was associated with increased levels of SB in lung tissue, whereas there was no difference in the hydrogen peroxide content in BALF (Kim et al. 2012). The same authors also showed that nose-only inhalation (0.17–0.96 mg/m^3, 6 h/days, 5 days/week for 28 days) of MWCNTs with a length of 330 nm was associated with increased levels of SB in lung tissue and elevated levels of hydrogen peroxide in BALF, whereas there was no sign of pulmonary inflammation (Kim et al. 2014). It should be emphasized that the concentration of hydrogen peroxide was quite low (only about 1 μM) and it usually requires higher concentrations of this oxidant to produce increased levels of SB in cultured cells (Collins et al. 1997; Singh et al. 1991). A direct connection between increased levels of hydrogen peroxide in BALF and SB levels in lung tissue thus seems implausible.

Exposure to MWCNTs by intraperitoneal (i.p.) injection in mice (0.25–0–75 mg/kg once a day for 5 days) was associated with increased levels of SB in peripheral blood leukocytes at 24 h after the last exposure as well as increased ROS production ex vivo in bone marrow cells (Patlolla et al. 2010).

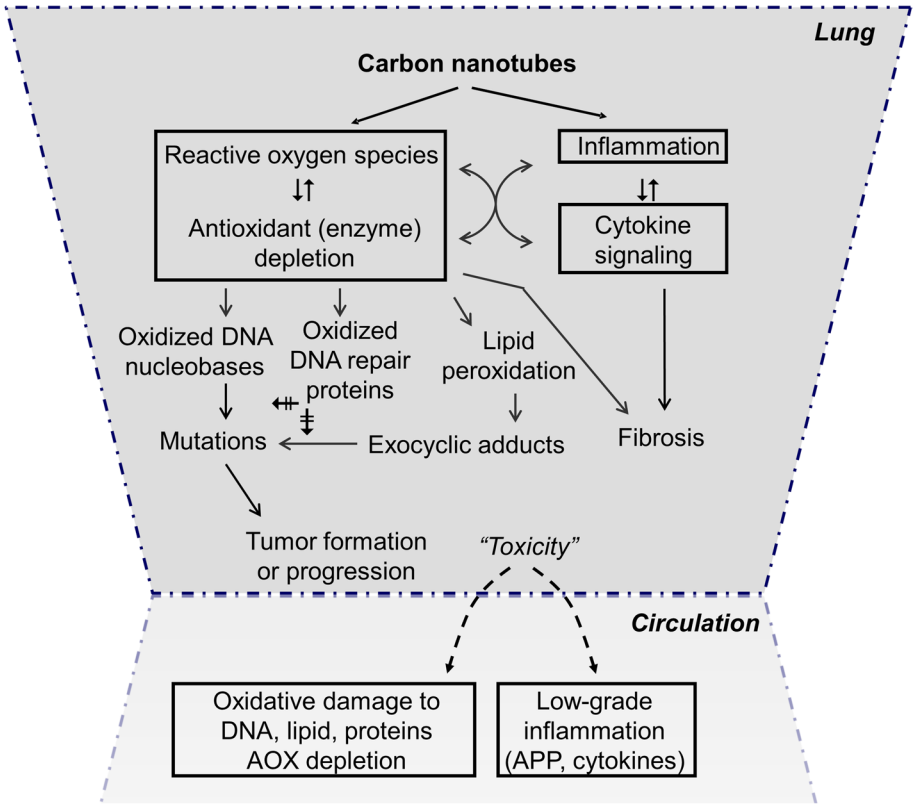

Fig. 4 The relationship between pulmonary exposure to carbon nanotubes and inflammation, oxidative stress, inflammation and lung diseases (fibrosis and tumors). The development of lung tumors may arise as a consequence of oxidative damage to DNA, aggravated by oxidative destruction of DNA-repair enzymes, leading to mutations and subsequent formation or progression of tumors. Inflammation reactions and oxidative stress may be measured in the circulation is experiments where lung tissue is not available as for instance human biomonitoring studies. *Abbreviations*: antioxidants (AOX) and acute phase proteins (APP)

Gastrointestinal administration of SWCNTs (0.064 and 0.64 mg/kg) was associated with increased levels of 8-oxodG in liver and lung tissue, whereas the same dose did not affect the level of 8-oxodG in colon mucosa cells (Folkmann et al. 2009). In the same tissue, there was unaltered OGG1-mediated DNA-repair activity and *Hmox-1* expression levels (Folkmann et al. 2009).

Collectively, there is compelling evidence showing an association between exposure to CNTs and elevated levels of oxidatively damaged DNA in cultured cells and animals, despite some methodological issues. However, it is clear that the majority of studies have used the comet assay. It would strengthen any conclusion regarding CNT-generated oxidative damage to DNA if other lesions were tested as well, such as lipid peroxidation-derived exocyclic adducts. Additionally, it is worthwhile mentioning that CNT exposure is associated with development of mutations and chromosomal changes (Gonzalez et al. 2009; Kumar and Dhawan 2013; van Berlo et al. 2012), which could occur by either oxidative stress-generated damage on DNA or other mechanisms. Figure 4 summarizes the association between pulmonary exposure to CNTs and genotoxicity in lung tissue. Based on this scheme, the exposure to CNTs promotes a pro-inflammatory and pro-oxidant milieu within the lungs, giving rise to oxidation of DNA and mutations. These events may culminate in the development of cancer. In humans, peripheral mononuclear blood cells are typically used as surrogate for pulmonary cells (Møller and Loft 2010). Although the mechanisms are not fully elucidated, it is possible that peripheral mononuclear blood cells come in contact with a pro-oxidant and pro-inflammatory environment in the pulmonary circulation or that pulmonary toxicity promotes a systemic low-grade inflammation response.

Lipid peroxidation products

Free radical attacks on poly-unsaturated or acyl-chains of the cellular lipid membrane can generate a range of lipid peroxidation products. Some of these are unstable fatty acyl peroxide radicals (typically described as "lipid hydroperoxides"), whereas others are more stable non-radical products (e.g., malondialdehyde, 4-hydroxynonenal and isoprostanes). Lipid peroxidation products are relevant in studies on atherosclerosis, although these compounds may also give rise to etheno- and propano-exocyclic rings on DNA nucleobases that are highly mutagenic (Winczura et al. 2012).

Several methods exist for measurements of lipid peroxidation products; some of these are widely used because they are technically very simple, but they are also highly unspecific with regard to being biomarkers of lipid peroxidation. Lipid hydroperoxides represent an early event of

lipid peroxidation as compared to detection of secondary products such as malondialdehyde. With regard to studies on CNT-generated lipid peroxidation, the measurements of lipid hydroperoxides have typically been carried out with a colorimetric assay that is available as a commercial kit (Inoue et al. 2010; Ravichandran et al. 2010; Srivastava et al. 2011). Increased levels of pulmonary inflammation and lipid hydroperoxides (measured by a commercial kit) in lung tissue were observed after repeated exposures to a SWCNTs with 23 % Fe (50 μg/week for 6 week) in a model for allergic airway inflammation, whereas a material type with 0.05 % Fe displayed no effect on lipid peroxidation products (Inoue et al. 2010). In addition, exposure to relatively high doses of aerosolized CNTs by nose-only inhalation (5 mg/kg per day delivered as 20 min/day for 7 consecutive days) increased the level of lipid hydroperoxides in lung tissue lysate from mice (Ravichandran et al. 2010). Using i.p. as route of administration, it was shown that mice liver tissue had increased level of lipid hydroperoxides after a 5-day exposure period to 0.25–0.75 mg/kg/day of functionalized MWCNTs (Patlolla et al. 2011). It has also been shown that SWCNTs with different content of iron (0.23 and 26 %) increased the levels of lipid hydroperoxides in RAW 264.7 cells, which were partly inhibited by pre-treatment with catalase (Kagan et al. 2006).

The measurement of malondialdehyde assessed by spectrophotometric detection of thiobarbituric reactive substances (TBARS) after short-term heating of the reaction mixture is very simple, but thiobarbituric acid reacts with a range of other molecules (e.g., glucose, deoxyribose, ascorbic acid, homocysteine, proline, glutamate and arginine), and there are dietary sources of malondialdehyde (Spickett et al. 2010). Indeed, it is recommended that the TBARS assay should not be used for assessment of lipid peroxidation in cells or animals (Halliwell and Whiteman 2004). A number of studies have used this assay of questionable quality in studies on lipid peroxidation events in cultured cells after exposure to CNTs, all of which have shown increased level of TBARS (Alarifi et al. 2014; Aldieri et al. 2013; Chen et al. 2011; Han et al. 2012; Pichardo et al. 2012; Reddy et al. 2010, 2011; Shvedova et al. 2003). However, exposure to MWCNTs (20 μg/mouse) by pharyngeal aspiration, with or without ozone inhalation (0.5 ppm for 3 h), did not increase the level of TBARS in serum, BALF or lung tissue (Han et al. 2008). A recent inter-laboratory validation trial documented that the measurement of malondialdehyde by HPLC was a reliable biomarker assay for lipid peroxidation products in samples from humans (Breusing et al. 2010). Using this type of assay, it was shown that exposure to SWCNTs increased the levels of malondialdehyde-TBA adducts in aortic endothelial cells, although there is uncertainty about the number of replicates in that study (Cheng et al. 2012).

Isoprostanes are secondary products of lipid peroxidation. It has been shown that isoprostanes measured by mass spectrometry is reliable as biomarker of lipid peroxidation (Kadiiska et al. 2005). However, the measurement of isoprostanes by antibody-based methods has been more commonly used, although the reliability of this method has been questioned (Halliwell and Whiteman 2004). Using this antibody-based method, it was shown that repeated exposures to MWCNTs (25.6 μg/week for 5 weeks by i.t. instillation) in rats were associated with elevated levels of 8-isoprostanes in lung tissue, whereas there were unaltered levels in BALF and serum (Cao et al. 2014). In addition, exposure to MWCNTs (20 μg/mouse) by pharyngeal aspiration, with or without ozone inhalation (0.5 ppm for 3 h), did not increase the level of 8-isoprostanes in lung tissue, BALF or serum of mice (Han et al. 2008).

Collectively, there is evidence indicating an association between exposure to CNTs and elevated levels of lipid peroxidation products in cell culture and lungs of animals after pulmonary exposure. Nevertheless, it should be emphasized that a substantial number of these observations have been documented with the use of non-optimal assays for lipid peroxidation products.

Pulmonary health outcomes after exposure in animal models

The majority of investigations in animal models have explored the effects of pulmonary exposure, either by inhalation or instillation of materials. However, CNTs may also reach the pleural and extrapulmonary tissues after inhalation or aspiration (Mercer et al. 2010, 2013b; Ryman-Rasmussen et al. 2009a). Certain studies have used i.p. injection of CNTs as a surrogate tissue for toxic effects to the mesothelial cells. Utilizing the peritoneal cavity as surrogate for the mesothelial lining of the lungs, it was shown that long MWCNTs was associated with increased influx of PMNs, frustrated phagocytosis and development of granuloma after administration of 50 μg/mouse as a single dose (Poland et al. 2008). The mechanism by which long CNTs may cause mesothelioma has been hypothesized to be via blockage of the lymphatic drainage holes (stomata) in the parietal pleural lumen. The accumulation of fibers is associated with a local tissue reaction, inflammation response (including frustrated phagocytosis) and a pro-oxidant milieu (Donaldson et al. 2010).

A study comparing different lengths of MWCNT administered by i.t. spraying every 2 weeks for 24 weeks in rats showed that the long type induced more pleural inflammation and mesothelial proliferation (Xu et al. 2014). In addition to prolonged inflammation, direct genotoxicity, tumor promotor and co-carcinogen activity have been described

as important mechanisms for carcinogenicity of man-made synthetic vitreous fibers and may also have relevance for CNTs (Hesterberg and Hart 2001).

Exposure to CNTs by the gastrointestinal tract or i.v. injection has only been investigated in few studies. However, regardless of the way of exposure, inflammation and oxidative stress have been explored or discussed as possible intermediate effects leading to toxic effects in target tissue. These studies can be segregated into investigations on effects in lung tissue or systemic responses (e.g., vascular effects or oxidative stress/inflammation responses in organs other than the lung).

Fibrosis in lungs after pulmonary exposure

A number of studies have investigated the effect of pulmonary exposure to CNTs on the lungs, especially with respect to development of fibrosis (a scarring in the lungs where patients have shortness of breath, chronic dry coughing, chest discomfort and fatigue). This condition is well-characterized from occupational exposure to asbestos.

Fibrosis was observed at 60 days post-exposure after i.t. instillation of MWCNTs (0.5–2 mg/rat). These doses were sufficiently large to produce pulmonary inflammation (assessed as increased number of PMNs and TNF in BALF) at day 3 after the instillation (Muller et al. 2005). This was supported by observations from an investigation in mice, showing granuloma formation and fibrosis at day 30 after a single exposure by oropharyngeal aspiration (1–4 mg/kg) of MWCNT (Wang et al. 2011). Granulomatous changes and bronchoalveolar hyperplasia, as well as interstitial collagen staining, were also observed after 13 weeks of inhalation of MWCNTs (0.1–6 mg/m^3, 6 h/day, 5 days/week) (Pauluhn 2010). Another study showed that inhalation of MWCNTs (2.5 mg/m^3, 6 h/day, 5 days/week for 13 weeks) only led to formation of multi-focal granulomatous inflammation with fibrotic changes in rats, although there was no sign of diffuse pulmonary fibrosis as judged by standard hematoxylin and eosin staining (Ma-Hock et al. 2009). Pulmonary inflammation, type II cell hyperplasia, microgranuloma formation and fibrosis were observed at day 91 after i.t. instillation of MWCNTs (160 μg/rat), whereas there was no effect at a dose of 40 μg/rat (Aiso et al. 2010). Likewise, inhalation of MWCNTs (5 or 10 mg/m^3, 5 h/day for 2–12 days) was associated with pulmonary fibrosis (Mercer et al. 2013a; Porter et al. 2013). However, there are also observations indicating that inhalation of relatively long MWCNTs (5–15 μm; 0.3–5 mg/m^3 per day, 6 h/day for 7 or 14 days in mice), i.t. instillation (0.5–20 μm; 1–100 μg/rat) or inhalation/instillation (10 μm; 50 μg/mouse for i.t. instillation) were not associated with granuloma formation and fibrosis (Elgrabli et al. 2008; Li et al. 2007a; Mitchell et al. 2007).

The link between exposure to SWCNTs, oxidative stress, inflammation and development of fibrosis has been exploited by Shvedova and co-workers in animal models. The first set of experiments showed that pharyngeal aspiration of SWCNTs (10 μg/mouse) with low iron content led to increased pulmonary inflammation, lipid peroxidation and depletion of GSH in BALF (most pronounced at day 1 after exposure) and progressive development of fibrosis over a 28-day post-exposure period (Shvedova et al. 2005). Inhalation of SWCNTs with 18 % iron content (5 mg/m^3, 5 h/day for 4 days) also decreased the GSH level and increased levels of protein thiols and malondialdehyde in lung tissue of mice (Shvedova et al. 2008a). The role of inflammation and oxidative stress was explored in knock-out models and antioxidant supplementation. It was shown that pharyngeal aspiration of SWCNTs (40 μg/mouse) in NADPH oxidase-deficient mice mounted a higher inflammatory response (PMNs, IL6, CCL2 in BALF and MPO activity in lung tissue) as compared to wild-type mice, whereas they had little collagen deposition and low levels of the pro-fibrotic cytokine TGF-β (Shvedova et al. 2008b). Another study showed that pharyngeal aspiration of SWCNTs (40 μg/mouse) decreased α-tocopherol, GSH and ascorbate levels at days 1, 7 and 28 after exposure (Shvedova et al. 2007). A vitamin E-deficient diet increased the level of pulmonary inflammation (PMNs, IL6 and TNF in BALF), pro-fibrotic response and deposition of collagen (Shvedova et al. 2007). The exposure to SWCNT (40 mg/mouse) by pharyngeal aspiration was associated with pulmonary fibrosis, inflammation and increased level of 4-hydroxynonenal and protein carbonyls in lung tissue at days 1, 7 and 28 post-exposure (Murray et al. 2012).

Carcinogenic responses

There has been considerable interest in carcinogenicity following exposure to CNTs. The development of mesothelioma following exposure to CNTs has been hypothesized, based on the resemblance to long (i.e., more than ten- to twenty-μm-length fibers) asbestos and vitreous fibers (Donaldson et al. 2006). However, there was no development of mesothelioma or other tumors in the peritoneal cavity after injection of MWCNTs with a fiber length of 700 nm on average (2 or 20 mg/rat), although the high dose was associated with inflammation as assessed by increased number of neutrophils in the peritoneal cavity at 24 h after the exposure (Muller et al. 2009). Another study showed granuloma formation, but not mesotheliomas, after implantation of SWCNT or MWCNTs in gelatin capsules into a peritoneal envelope (25 mg/kg), although it should be emphasized that the group size (six rats/group) and exposure (12 months) might not be optimal for detection of

mesotheliomas by CNTs in wild-type animals (Varga and Szendi 2010).

Utilizing the peritoneal cavity as surrogate for the mesothelial lining of the lungs, it was observed that i.p. injection of 3 mg/mouse (or 120 mg/kg) of MWCNTs was associated mesothelioma development and mortality in *p*53 heterozygous mice at day 180 after the exposure, although the relevance of the observations was questioned because of the high dose, poor material characterization and histological assessment of effects (Takagi et al. 2008). However, a later study showed a dose-dependent (3–300 μg/mouse by i.p. injection) induction of mesothelioma at 12 months after the administration in *p*53 heterozygous mice (Takagi et al. 2012). It was also demonstrated that i.p. injection of thin MWCNTs (diameter = 50 nm, 1–10 mg/kg) with high crystallinity was associated with development of mesotheliomas, whereas thick (diameter = 150 nm) or tangled MWCNTs had no effect in terms of carcinogenicity (Nagai et al. 2011, 2013). Intrascrotal injection of MWCNTs in rats (0.26 mg/rat or 1 mg/kg) was also associated with increased incidence of mesothelioma during an observation period of 52 weeks (Sakamoto et al. 2009).

Using a more realistic and relevant exposure model and dose regiment showed that inhalation of MWCNTs (5 mg/m^3, 5 h/day for 15 days, estimated lung burden 31 μg/mouse) increased lung tumor burden, including adenocarcinomas and sarcomatous mesotheliomas, in methylcholanthrene pre-exposed mice (Sargent et al. 2014). This observation demonstrates that MWCNTs possessed the ability to stimulate both growth and progression of tumors.

Collectively, the data indicate that exposure to CNTs is associated with pulmonary fibrosis and possibly also increased risk of cancer.

Systemic effects on the vascular system in animals after exposure to CNTs

Cardiovascular events in humans (e.g., myocardial infarction) are usually observed as sudden aggravation of a chronic disease progress, which may have been unnoticed by the patient. In particular, atherosclerosis is a progressive disease with accumulation of lipids and fibrous material in the intima of mainly medium-sized and large arteries, promoted by a pro-inflammatory milieu and oxidative stress in endothelial cells and intima of blood vessels (Hansson and Libby 2006). The endothelium is the monolayer of cells that covers the inner surface of blood vessels. They are involved in the regulation of blood flow as well as maintenance of the vascular wall in a quiescent state by inhibition of inflammation, cellular proliferation and thrombosis (Hansson and Libby 2006). The exposure to particles causes vascular effects by promotion of vasomotor dysfunction, atherosclerosis, increased thrombosis tendency and arrhythmia, of which systemic oxidative stress and inflammation are considered important mediators (Langrish et al. 2012).

The studies on effect on the vascular system after exposure to CNTs have focused on both external (e.g., airways related to occupational exposure) and direct systemic administration (e.g., i.v. injection of CNTs related to nanomedicine). Therefore, the administered systemic doses can be rather high from the perspective of pulmonary exposure, assuming low fraction of translocation from the lungs, although it can be relevant with regard to nanomedicine.

Vasomotor dysfunction

Optimal blood flow to organs and cells is achieved by dilation or constriction of blood vessels. Imbalance between vasodilation and vasoconstriction is the hallmark of endothelial dysfunction, although decreased release of plasminogen activator factor from the endothelium is also used as marker of endothelial dysfunction (Deanfield et al. 2005; Heitzer et al. 2001; Widlansky et al. 2003). This is considered to be one of the earliest events in the formation of an atheroma and the magnitude of endothelial dysfunction correlates with the extent of atherosclerosis (Ross 1999). In the non-pathological condition, endothelial cells generate nitric oxide (NO) by endothelial nitric oxide synthase (eNOS), which can be stimulated by vasorelaxing agents such as acetylcholine or bradykinin (Vanhoutte 2009). NO diffuses to the smooth muscle cells and promotes the relaxation of the vessel. However, endothelial dysfunction is associated with an uncoupling of eNOS, leading to increased production of superoxide anion radicals and reduced NO production (Laursen et al. 2001). NO can react with superoxide anion radicals to form peroxynitrite, which is a highly proficient oxidizing species. The net result is oxidative stress and a blunted vasodilation response in the blood vessels, which can be accompanied by increased supply of vasoconstricting compounds or an augmented response. The vasomotor function is assessed on intact vessel segments, either in vivo or ex vivo, as the dilation/contraction responses require communication between endothelial cells and smooth muscle cells. Essentially, dysregulation of the vasomotor function in particle-exposed humans or animals manifests as a blunted response to relaxation stimuli and/or enhanced response to contraction responses (Møller et al. 2011).

Inhalation of MWCNT (5 mg/m^3 for 5 h and sacrifice at 24–168 h after the exposure) was associated with pulmonary inflammation (increased number of PMNs in BALF) and attenuated vasodilatation response in subepicardial arterioles of rats, whereas there was unaltered endothelium-independent vasodilation, vasoconstriction and myogenic

response to pressurized vessels (Stapleton et al. 2012). These observations indicate a decreased bioavailability of NO, leading to blunted endothelium-dependent vasodilation. Another study showed unaltered ex vivo endothelium-dependent vasodilation in aorta after i.t. instillation of SWCNT (0.5 mg/kg administered at 26 and 2 h before sacrifice), which was a dose that was not associated with oxidative stress and inflammation in the lungs (Vesterdal et al. 2014b). In addition, a single i.t. instillation of MWCNTs (100 μg/rat) neither affected the ex vivo endothelial-dependent nor the independent vasorelaxation in coronary arteries, whereas there was a trend toward increased vasoconstriction (Thompson et al. 2014).

The endothelial and vasomotor dysfunction may be associated with increased resistance of blood flow and associated with higher cardiac workload. Intratracheal instillation of SWCNT (0.6 mg/rat) in spontaneous hypertensive rats was associated with a thickening of arterial vessels, toxic responses to the heart (i.e., edema and leakage of erythrocytes and myofibril degeneration) and vascular responses (endothelin-1, angiotensin I converting enzyme and fibrinogen), whereas there were unaltered serum levels of C-reactive protein, TNF, ICAM-1 and von Willebrand factor (Ge et al. 2012).

Atherosclerosis

The sequence of events leading to atherosclerosis is typically initiated with the expression of cell adhesion molecules on endothelial cells, including ICAM-1, VCAM-1, SELE and SELP. These cell adhesion molecules bind to monocytes and assist their migration from the lumen to the vessel wall. The early phase of atherosclerosis is characterized by formation of fatty streaks consisting of lipid-laden macrophages (foam cells) and lymphocytes beneath the endothelium. In later stages, there is enlargement of foam cells and development of a necrotic core, which is associated with protrusion of the endothelium into the blood vessel lumen. Narrowing of blood vessel lumen may subsequently be associated with outward remodeling (i.e., compensatory structural rearrangement of the vessel wall to sustain blood flow) or decrease in blood flow. The final clinical manifestation of atherosclerosis is plaque rupture, leading to myocardial infarction.

The exposure to SWCNT (20 μg/mouse) by i.t. instillation every other week for 8 weeks increased the atherosclerotic lesion area in aorta and brachiocephalic artery of *ApoE* knockout mice fed a high-fat diet, whereas there was no difference between SWCNT-exposed mice and controls in the group of mice on a regular diet (Li et al. 2007b). The atherosclerotic plaques in the brachiocephalic artery was accompanied by increased protein expression of MAC-3 (a marker of macrophages and monocytes) and VCAM-1 expression, whereas plasma levels of CCL2, IL6, IL12, TNF and INFγ were unaltered (Li et al. 2007b). A later study showed that i.t. instillation of MWCNTs (25.6 μg/week for 5 weeks) increased the atherosclerotic plaque area in aorta, which was associated with increased pulmonary inflammation and oxidative stress, whereas systemic responses on cytokines (IL1β, IL4, IL6, IL12, IL13, G-CSF, KC, CCL2, MIP1β, RANTES and TNF) and 8-isoprostane were unaltered (Cao et al. 2014). Nevertheless, cell culture experiments showed that MWCNT-induced lipid accumulation in activated monocytic THP1 cells and this could be reduced by pre-treatment with NAC (Cao et al. 2014). Evidence of associations between exposure to MWCNTs and endothelial cell activation also comes from an in vitro study where MWCNTs exposure to human small airway epithelial cells activated endothelial cells across a Transwell barrier to secrete soluble VCAM-1 (Snyder-Talkington et al. 2013).

The i.v. administration of MWCNTs (50–200 μg/kg twice a week for 4 months) increased the level of aortic atherosclerosis in the rats on a high-fat diet (Xu et al. 2012).

CNT effects on the autonomic function of the vascular system

The assessment of autonomic dysfunction following pulmonary exposure to nanomaterials is only sparsely investigated. This effect on the vascular system is promoted by stimulation of vagal sensory nerves in the lung, leading to autonomic dysregulation and possibly cardiac arrhythmias (Mann et al. 2012). It has been shown that i.t. instillation of SWCNTs (1 mg/kg) was associated with altered autonomic cardiovascular regulation, documented by decreased number of baroreflex sequences in rats (Legramante et al. 2009).

Increased thrombosis

There was increased thrombus formation tendency in carotid arteries in vivo after photochemical injury in mice at 24 h after a single i.t. instillation of MWCNTs (200 or 400 μg/mouse), which also generated pulmonary inflammation (Nemmar et al. 2007). The pro-thrombosis tendency was inhibited by i.v. administration of SELP-neutralizing antibody prior to the pulmonary MWCNT exposure (Nemmar et al. 2007). In addition, intra-arterial injection of SWCNT (1 mg/kg) decreased the thrombosis time in mesenteric arteries and cremasteric arterioles of mice, which was in keeping with in vitro observations of accelerated platelet aggregation and increased expression of SELP (Bihari et al. 2010). Another study showed that injection of MWCNTs (150 μg/mouse) in the tail vein was associated with increased thrombosis tendency in blood collected from the same vein at 3, 24 or 48 h post-exposure

(Burke et al. 2011). Intra-arterial injection of SWCNTs shortened the thrombosis time in cremasteric arterioles, whereas the thrombosis tendency was unaffected in venules of mice (Holzer et al. 2014). Increased thrombosis was also observed in the carotid artery of rats after i.v. injection of CNTs in the femoral vein (25 µg/rat), and the same samples stimulated platelet aggregation in vitro (Radomski et al. 2005). Platelet aggregation is a process that is linked to ROS formation and calcium mobilization (Violi and Pignatelli 2012). Indeed, higher platelet aggregation has been shown to be related to CNT-facilitated extracellular calcium influx into platelets (Semberova et al. 2009).

Susceptibility to ischemia and reperfusion injury

Thrombus formation in a blood vessel leads to reduced or even blockage of blood flow. This is associated with reduced oxygen supply (ischemia) to the affected tissue, accompanied with a range of metabolic changes in endothelial cells. Upon restoration of the blood supply, reoxygenation of the affected tissue may lead to cytotoxicity or necrosis (reperfusion injury) that may culminate in substantial decrease in organ function as seen following stroke or myocardial infarction (Carden and Granger 2000). Oxidative stress plays an important role in ischemia and reperfusion injury by highly augmented ROS production from multiple endogenous sources such as xanthine oxidase, NADPH oxidase and mitochondrial dysfunction (Chen and Zweier 2014; Crimi et al. 2007; Kleikers et al. 2012). Increased susceptibility to ischemia and reperfusion injury following exposure to CNTs may be due to a strong thrombus formation, prolonged period to reperfusion or lower amounts of antioxidants in the tissue.

A single oropharyngeal aspiration of MWCNT (0.1–100 µg/mouse) was associated with increased susceptibility to cardiac ischemia/reperfusion injury at doses that did not promote pulmonary inflammation and there were unaltered levels of cytokines and leukocytes in the circulation (Urankar et al. 2012). Isolated hearts from MWCNT-exposed rats (100 µg/rat), tested in the Langendorff model, also demonstrated exacerbated infarct size after ischemia/reperfusion injury and an increased vasoconstriction response (Thompson et al. 2014). In addition, one study showed that oropharyngeal aspiration (10 or 40 µg/mouse) of SWCNTs had no effect on pulmonary inflammation (PMNs, IL6, MIP2 and TNF in BALF), systemic markers (CRP and fibrinogen), myocardial histology or infarct size following ex vivo ischemia and reperfusion injury (Tong et al. 2009). However, acid treatment of SWCNTs to increase dispersion and solubility was associated with pulmonary inflammation, myocardial degeneration (signs of small clusters of shrunken rounded, hypereosinophilic myocytes) and increased infarct size (Tong et al. 2009). Interestingly, administration of lipopolysaccharide produced a substantial influx of neutrophils in BALF, but the vascular and cardiac endpoints were unaltered, indicating that pulmonary inflammation per se may not mediate these systemic effects (Tong et al. 2009).

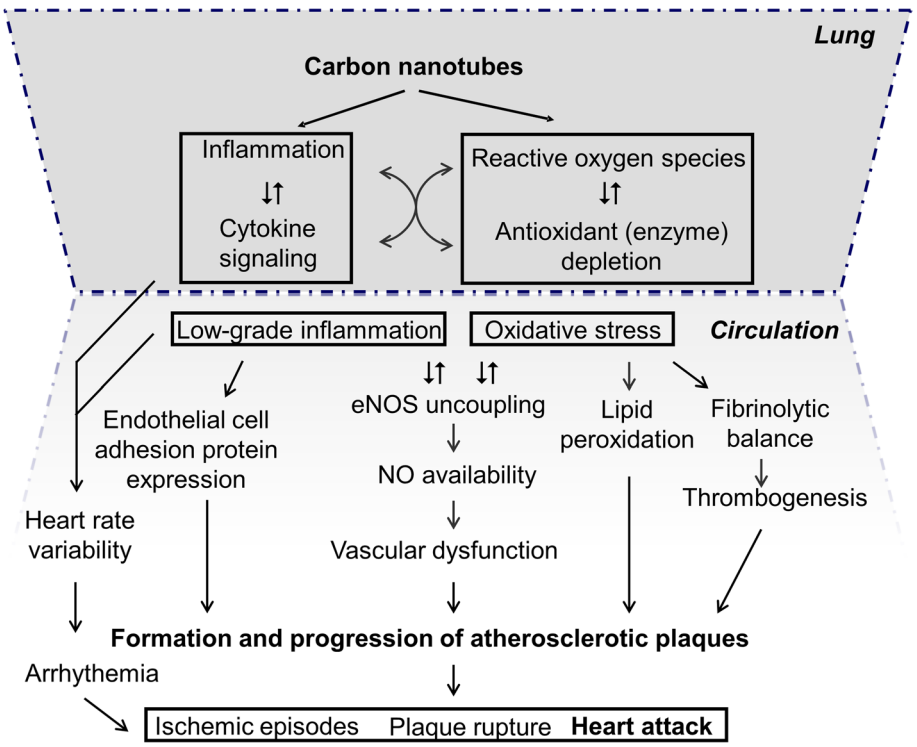

Fig. 5 Relationship between pulmonary exposure to carbon nanotubes, low-grade systemic inflammation, oxidative stress development of cardiovascular diseases. Carbon nanotube-mediated systemic inflammation and oxidative stress maybe associated with the same reactions in the lungs and affect the endothelial cell function and fibrinolytic balance, leading to formation or progression of atherosclerosis and ultimately heart attack

Collectively, the results indicate that exposure to CNT is associated with cardiovascular diseases in animals by mechanisms encompassing atherosclerosis and thrombosis, conferring increased risk of atherothrombosis and susceptibility to ischemic/reperfusion injury (Fig. 5). However, systemic oxidative stress mechanisms leading to intermediate vascular effects (atherosclerosis, vasomotor dysfunction and prothrombotic tendency) are not strongly supported by experimental evidence across studies. There are very few studies that directly link CNT-mediated oxidative stress to vascular endpoints by for instance demonstrating abolishment of effects by treatment with antioxidants.

Conclusions

The compilation of studies in the present review demonstrates that exposure to CNTs is associated with inflammation and oxidative stress, albeit there are some contrasting results. There are multi-factorial causes of these differences in effect, including differences in CNT characteristics, dispersion protocols, cell types, animal strains and assays for the measurement of oxidative stress and inflammation endpoints. As studies typically differ by several factors, it is difficult to pinpoint which of these factors are most important for causing the toxicological effects. However, collectively there is evidence showing elevated levels of oxidized lipids and DNA nucleobase products in both animal tissues and cells following exposure to CNTs. There are only few studies on ROS production in tissue homogenate, whereas increased ROS production in cultured cells has been widely documented with the DCFH assay. The same assay has also been used to demonstrate acellular ROS production, whereas ESR measurements indicate no ROS production. Intracellular sources of ROS include enzymes such as NADPH oxidase, which may be activated by inflammation signaling. Evidences of cellular oxidative stress also come from observations of decreased levels of endogenous antioxidants and upregulation of Hmox-1 levels. Importantly, supplementation with antioxidants can attenuate the ROS production and inflammation signaling. These observations place oxidative stress as a central mechanism in cellular and tissue damage, although it should also be acknowledged that non-oxidative stress-mediated cellular damage may occur by direct interaction between CNTs and biomolecules (Shvedova et al. 2012).

There is substantial evidence of fibrosis following pulmonary exposure to CNTs. Currently, the evidence of increased risk of cancer is principally provided from non-physiological routes of exposure such as i.p. injection as a model for pleural exposure. It remains to be determined whether direct CNT-mediated ROS production occurs or if frustrated phagocytosis of long CNTs produces a pro-oxidant milieu. Interestingly, pulmonary exposure to CNTs is associated with accelerated progression of atherosclerosis and pro-thrombotic tendency in the vasculature, although the mechanisms behind these effects remain to be thoroughly assessed. Finally, oxidative stress seems to occur concurrently with vascular effects.

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