Arch Toxicol (2011) 85:733–741
DOI 10.1007/s00204-010-0546-4

REVIEW ARTICLE

Nanoparticles: molecular targets and cell signalling

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Received: 22 February 2010 / Accepted: 19 April 2010 / Published online: 26 May 2010

Abstract Increasing evidence linking nanoparticles (NPs) with different cellular outcomes necessitate an urgent need for the better understanding of cellular signalling pathways triggered by NPs. Oxidative stress has largely been reported to be implicated in NP-induced toxicity. It could activate a wide variety of cellular events such as cell cycle arrest, apoptosis, inflammation and induction of antioxidant enzymes. These responses occur after the activation of different cellular pathways. In this context, three groups of MAP kinase cascades [ERK (extracellular signal-regulated kinases), p38 mitogen-activated protein kinase and JNK (c-Jun N-terminal kinases)] as well as redox-sensitive transcription factors such as NFκB and Nrf-2 were specially investigated. The ability of NPs to interact with these signalling pathways could partially explain their cytotoxicity. The induction of apoptosis is also closely related to the modulation of signalling pathways induced by NPs. Newly emerged scientific areas of research are the studies on interactions between NPs and biological molecules in body fluids, cellular microenvironment, intracellular components or secreted cellular proteins such as cytokines, growth factors and enzymes and use of engineered NPs to target various signal transduction pathways in cancer therapy. Recently published data present the ability of NPs to interact with membrane receptors leading to a possible aggregation of these receptors. These interactions could lead to a sustained modulation of specific signalling in the target cells or paracrine and even "by-stander" effects of the neighbouring cells or tissues. However, oxidative stress is not sufficient to explain specific mechanisms which could be induced by NPs, and these new findings emphasize the need to revise the paradigm of oxidative stress to explain the effects of NPs.

Keywords Nanoparticle · Oxidative stress · Cell signalling · Inflammation · Nano–bio interactions · Apoptosis

This article is published as part of Special Issue on Nanotoxicology.

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Introduction

Nanoparticle-induced toxicological mechanisms have become one of the most studied topics in toxicology during the last few years and are the subject of huge debates. In 1990s, toxicological studies began to seek biological plausibility for the epidemiological findings of the association between health effects and ambient fine particle concentrations (Brunekreef and Holgate 2002; Pope III et al. 1999). In vitro and in vivo studies on fine and ultrafine airborne particles such as diesel exhaust particles, PM2.5, PM1 and carbon black have lead to consider their central role in the adverse health effects of atmospheric particles (reviewed in Donaldson et al. 2005). Furthermore, in vivo (Oberdorster et al. 2000; Brown et al. 2001; Wilson et al. 2002; Renwick et al. 2004) and in vitro studies (Rahman et al. 2002; Brown et al. 2004; Kang et al. 2008) comparing the toxicity of NPs (less than 100 nm of diameter) with their fine counterparts of the same chemical composition have enlightened the higher toxic potentials of NPs and their ability to induce an inflammatory response. Same mass of poorly soluble or

insoluble NPs such as titanium dioxide (TiO_2) and carbon black (CB) induce pulmonary inflammation after inhalation or intratracheal instillation in rodents proportionate to their surface area (Duffin et al. 2007; Stoeger et al. 2006; Oberdorster et al. 2005). Surface area (which increases many folds compared to the same weight of the micrometre-sized compound) and surface reactivity are taken as the principal indicators of NP reactivity as it has been shown that cells and organs can demonstrate a toxic response even to apparently non-toxic substances when they are exposed to a sufficient dose in the nanometre size range (Donaldson et al. 2005). All these observations are the roots of the development of a new field of toxicology, nanotoxicology, defined by Donaldson et al. in 2004 and Oberdorster et al. in 2005. Rapidly, it appeared that the comprehension of the cellular and molecular mechanisms, which lead to the biological effects of NPs, was essential to develop safe nanomaterials and accurate assays for risk assessment of engineered NPs (Oberdorster et al. 2005). Recently, an increased number of reviews have summarized the current knowledge on nanotoxicology (Johnston et al. 2010; Borm et al. 2006a; Borm and Muller-Schulte 2006; Borm et al. 2006b; Oberdorster 2010; Oberdorster et al. 2005; Stone et al. 2009). Some were focused on the demonstrated or hypothetic cellular mechanisms and were useful to develop researches on these responses (Nel et al. 2006; Unfried et al. 2007).

The uptake of NPs by target cells like macrophages or epithelial cells plays a central role in the biological responses such as direct or indirect production of ROS, which occurs in relation to size, chemical composition and surface reactivity of the NPs. The interaction with cell membranes and receptors is strongly associated with the ability of NPs to associate biological molecules from the cellular microenvironment and body fluids, thus forming a protein corona (Nel et al. 2009). It becomes obvious that the NPs do not interact directly with the cells but the protein coronas of NPs play an essential role in the interaction with lipids or protein receptors of the cell membrane (Lynch et al. 2009; Hellstrand et al. 2009; Lundqvist et al. 2008). The NP surface and its specific chemical compounds resulting from the engineering processes, the methods used for dispersion and experimental preparation determine the ability to adsorb specific biological compounds especially proteins. This corona is important for the uptake and could lead to the activation of specific signalling pathways in relation to cellular differentiation. The signalling cascades specifically induced by ultrafine particles or NPs have been, in a first step, mostly studied in lung cells which are the main target. Diesel exhaust particles (DEP) were largely used as models of fine and ultrafine airborne particles and compared to PM 2.5 and PM 1 as well as engineered NPs (Baulig et al. 2003; Rumelhard et al. 2007). All these particles have very different chemical patterns and differential abilities to form agglomerates. However, it appeared that common responses could be detected, and the paradigm of the central role of oxidative stress was developed (Li et al. 2003; Ayres et al. 2008; Nel et al. 2006; Xia et al. 2006). The activation of pathways, nuclear factors and specific genetic programs depend directly or indirectly on the level of ROS production outside or inside the cell (Fig. 1). Oxidative stress could lead to cell death by necrosis or apoptosis or adaptive responses including pro-inflammatory responses, antioxidant enzyme activations, repair processes, effects on cell cycle control and proliferation. However, new insights during the last year have pointed out other specific effects of NPs related to their ability to interact with membrane receptors leading to a possible aggregation of these receptors. These interactions could lead to a sustained modulation of specific signalling pathways in the target cells and contribute to the development of diseases but could also be of use to develop therapeutic strategies.

The induction of apoptosis is also closely related to the modulation of signalling pathways induced by NPs. These molecular mechanisms were well studied in recent papers which showed that several pathways could be involved depending on the size and the chemical composition of NP.

At last, we will analyse the possible interaction of NPs with the cell cycle and, especially, with mitosis. Recently, it has been shown that TiO_2 NP could disturb mitotic progression and chromosome segregation after long-term exposure via the ERK signalling and production of ROS. This could lead to chromosomal instability and cell transformation.

Interactions of nanoparticles with cell membranes and membrane receptors

Several studies reviewed in Nel et al. (2009) suggest that binding of NPs to proteins may determine their interaction with cells and tissues in vivo. In addition, interaction of NPs with cell surface proteins such as receptors may induce biological responses (Mailander and Landfester 2009). These NP-protein interactions are of prime importance since they may have biomedical and/or toxicological relevance. Taking advantage of these properties, NPs bearing surface-conjugated protein ligands have been developed for a variety of biomedical applications since multivalent conjugation of targeting protein ligands on the surface of the NPs may enhance specific binding to a desired cellular target (Tassa et al. 2010). Many of these studies rely on the binding of a protein ligand-coated NPs to cellular receptors. For instance, it has been shown that protamine-based NPs coated with the vasoactive intestinal peptide (VIP) can specifically target tumour cells expressing the VPAC1 and VPAC2 receptors (Ortner et al. 2010). However, no data are available on the biological consequences of the binding

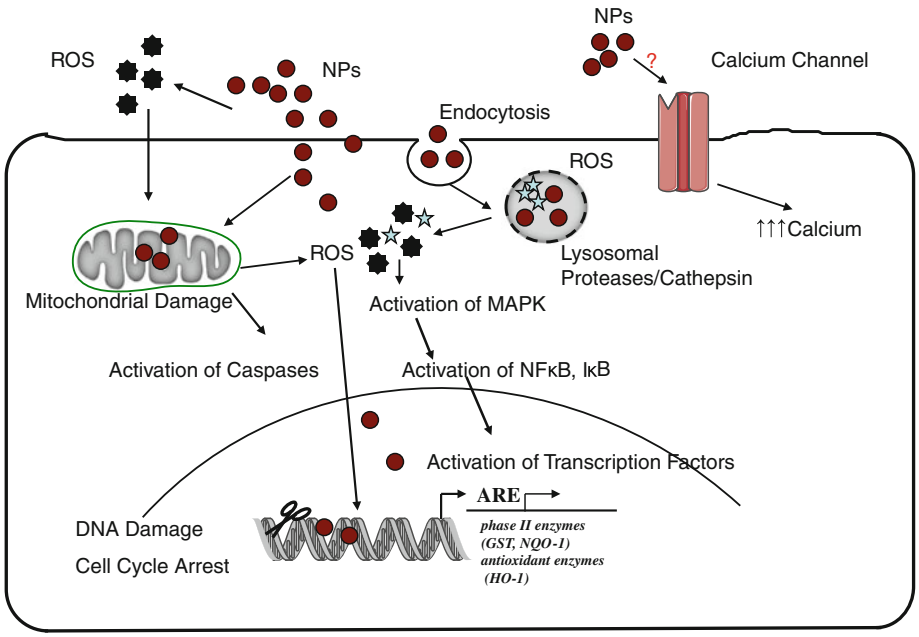

Fig. 1 A simplified illustration of NP-triggered cellular pathways and implication of oxidative stress in these responses. ROS produced by NPs in immediate cellular environment or inside the cells lead to activation of stress-dependent signalling pathways like MAPK or IKK, which ultimately result in the activation of transcription factors e.g. AP-1, NF-kB or Nrf2 and altered gene expression via e.g. the antioxidant response element (ARE).Oxidative stress also results in the damage to different organelles like the mitochondria, the lysosomes and the nucleus. These further amplify the stress signal through different mediators (caspases, calpains, cathepsins) resulting in DNA fragmentation and apoptosis. Accumulation of high intracellular calcium levels might also act as an alternative mechanism for the induction of these mechanisms

of VIP nanoparticles to the cellular VPAC receptors. Wagner and colleagues have shown enhanced drug targeting by coupling an anti-$\alpha_5\beta_3$ integrin antibody to albumin NPs (Wagner et al. 2010). Integrins such as $\alpha_5\beta_3$ are known to play a key role in cell signalling, and their activation by extracellular ligands such as antibodies can impact biological processes such as matrix remodelling, angiogenesis, tissue differentiation and cell migration (Harburger and Calderwood 2009). Although this study does not address the putative activation of the $\alpha_5\beta_3$ integrin by the antibody–nanoparticle complex, one cannot rule out a signalling effect of the nanoparticle–protein complex. Interestingly, it has been shown that CB nanoparticle-induced lung epithelial cell proliferation is due at least in part to β1-integrin activation (Unfried et al. 2008). Huang et al. reported interesting data on receptor aggregation induced by NPs leading to cell signalling (Huang et al. 2009b). Indeed, complex cell mechanisms can be triggered by multivalent ligands that first bind to membrane receptor and then promote receptor clustering and subsequent intracellular signal transduction. The authors showed that gold NPs coated with dinitrophenyl at a controlled density were able to bind and cross-link IgE-Fc epsilon receptors leading to degranulation and consequent release of chemical mediators by rat basophilic leukaemia cells (Huang et al. 2009b). In addition, activation of cell signalling pathways by interaction of magnetic NPs with proteins on the cell surface is increasingly studied in particular for mechanosensitive cell receptors (Hughes et al. 2005). To this end, magnetic NPs are coated with specific ligands that enable them to bind specifically to receptors on the cell surface. Application of a magnetic field pulls on the NPs and leads to the delivery of nanoscale forces at the ligand–receptor bond, thus inducing mechanotransduction pathways. Other studies used integrin ligands such as fibronectin bound to magnetic NPs (Sniadecki 2010).

In addition to these rationally designed interactions between NPs and cell surface proteins for biotechnological applications, increasing evidence indicate that in biological fluids proteins associate with NPs to form a “corona” (Fig. 2). The amount and the structural/functional properties of the adsorbed proteins shape the interactions of these nanomaterials with the cells and potentially contribute to their biological responses (Cedervall et al. 2007b). Recent studies have clearly identified a number of serum proteins that bind to CB, TiO_2 or acrylamide NPs (Cedervall et al. 2007a; Deng et al. 2009; Val et al. 2009). Among the proteins identified, several such as apoliprotein E, granulocyte–macrophage colony-stimulating factor (GM-CSF) or transferrin are ligands for cellular receptors. Although the structural and functional status of these proteins absorbed on the NP surfaces have not been addressed in these

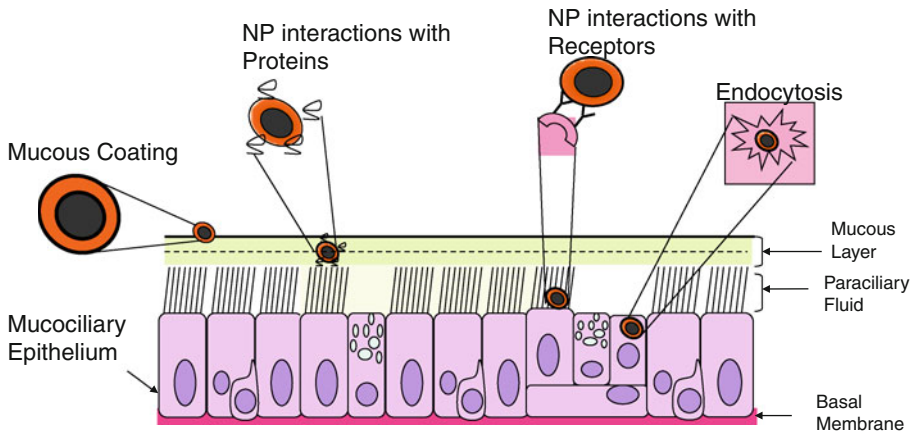

Fig. 2 Diagrammatic representation of interaction between the NPs and respiratory epithelial cells which is modified by the mucous coating, attachment of proteins from mucous and surfactant layers and interaction with cell surface receptors (Adapted from Pr. W. Kreyling)

studies, these proteins may contribute to the biological effects of NPs through activation/inactivation of receptor-dependent signalling.

These interactions may also play an important role in the uptake of NPs by various types of cells. It is likely that different cell types might have different uptake mechanisms, even for the same NPs. It has been postulated that NPs less than 100 nm can enter the cells, less than 40 nm can enter the cell nucleus and smaller than 35 nm can cross the blood–brain barrier (Dawson et al. 2009). But in the presence of obvious discrepancies in the recent literature about the optimal size, shape and mechanisms of internalization of NPs, this seems to be an oversimplified presentation of the actual scenario. The surface charge of the particle is an important factor for endocytosis, and it is expected that the positively charged NPs will show higher internalization than negatively charged NPs since the cell membranes are negatively charged giving higher possibilities of anchoring of positively charged NPs to the cell surface and thus favouring endocytosis. Using surface functionalized polymeric NPs with carboxyl and amino side groups, a clear correlation of surface charge and uptake has been shown for HeLa and Jurkat cell lines (Lorenz et al. 2006; Mailander and Landfester 2009). However, negatively charged NPs has also been shown to have enhanced uptake when compared to unfunctionalized NPs. This might be explained by their possible interactions with proteins. In these experiments, the authors demonstrated that endocytosis is energy dependent and is highly reliant on dynamin and F-actin. Tyrosine kinases located in the lipid rafts were also involved, suggesting a dynamin- and lipid raft-dependent uptake mechanism (Dausend et al. 2008). Phagocytosis is mostly involved in the endocytosis of large particles (more than 500 nm) and also in the uptake of the aggregates or agglomerates of NPs. Interaction of NPs with biological fluids leading to opsonisation could also promote phagocytosis of NPs (Dobrovolskaia and McNeil 2007). It has been proposed that macropinocytosis is an important mechanism for positively charged NPs internalization, and these vesicles are further transported in the cytoplasm of the cells through microtubule network (Dausend et al. 2008). Recently, it has been shown that aggregates or agglomerates of CB and TiO_2 NPs are internalized by macropinocytosis by cells in culture (Hussain et al. 2009; Singh et al. 2007).

Recent literature suggests that the size of the NPs determine caveolin- versus clathrin-dependent uptakes. However, the published data lacks concordance about the threshold diameter between these processes. In a study, it was found that NPs of less than 200 nm enter the cells through clathrin-coated pits, while bigger NPs are internalized by a caveolin-dependent endocytosis process (Rejman et al. 2004; Nel et al. 2009); whereas in another study, it was postulated that 50–80 nm are optimal diameters for caveolin-dependent endocytosis, while for clathrin-dependent endocytosis 120 nm was considered as optimal (Gratton et al. 2008). At least, it has been shown that cationic NPs could pass though cell membranes by generating transient holes without bilayer disruption (Gratton et al. 2008). This explains the presence of free NPs (without membrane covering) in the cytoplasm of the cells. Another possible explanation could be the release of NPs after rupture of endosomal compartment.

Nanoparticles, signalling pathways and cellular responses: what is new?

The paradigm of the central role of oxidative stress in cellular responses such as inflammation or apoptosis was first presented to be the main explanation of NPs toxicity (Nel et al. 2006). However, it is likely that it is not sufficient to explain all their biological effects (Donaldson et al. 2009).

Indeed, ROS play important roles in cells either by acting as second messengers leading to the activation of specific pathways and gene expressions or by causing cell

death. The oxidative stress results from an excess of ROS which overwhelms the antioxidant capacities of the cells. The hierarchical oxidative stress model (Nel et al. 2006; Xia et al. 2006) proposes that at a minor level of oxidative stress, the antioxidant protection is activated; whereas at a higher level, cell membrane and organelle injuries could lead to cell death, specific signalling pathways and gene expression being involved at each step. This model could explain the cell responses to numerous NPs such as TiO_2, CB, silica, Ag, magnetite, CeO_2 and WCo–Co (Xia et al. 2006; Hussain et al. 2009; Eom and Choi 2009; Kim et al. 2009; Park et al. 2008; Park et al. 2009; Hsin et al. 2008; Ding et al. 2009). The induction of oxidative stress is due to the inherent abilities of NPs to produce reactive oxygen species (ROS) especially owing to their chemical composition and interactions with the cellular components (Nel et al. 2006; Koike and Kobayashi 2006).

These diverse cellular responses occur after the activation of different cellular pathways. Three groups of MAP kinase cascades have especially been investigated: ERK, p38 and JNK in association with redox-sensitive transcription factors such as NFκB and Nrf-2. This last transcription factor plays an essential role in the antioxidant response element (ARE)-mediated expression of phase 2 enzymes such as NQO1 (NADPH quinone oxidoreductase-1) and antioxidant enzymes such as haeme-oxygenase-1 (HO-1). The activation of HO-1 is an interesting and new data on the effects of CeO_2 NP on human bronchial cells (BEAS 2B cell line). CeO_2 NPs induce a significant increase in cytosolic ROS leading to a strong induction of HO-1 via the p38-Nrf-2 signalling pathway (Eom and Choi 2009). However, the mechanisms which lead to the activation of this specific pathway were not explained and need further analysis. The response seems to be specific since no activation of the redox-sensitive transcription factor NFκB is observed at the same concentrations (neither nuclear localization of NFκB nor IκB cytosolic degradation) which is not classical since an oxidative stress response is usually associated with the activation of MAP Kinase pathways, NFκB and AP-1 activation followed by the transcription of many genes under the control of these two transcription factors such as pro-inflammatory genes (Lao et al. 2009). The pro-inflammatory response generally increases when the size of the NPs decreases (Hussain et al. 2009). The ability of NPs to interact with these signalling pathways could partially explain their cytotoxicity.

A scientific area has recently emerged from these studies, which is the use of engineered NPs that can target various signal transduction pathways in cancer. The MAPK signal transduction cascade is deregulated in a variety of human tumours. Basu et al. (2009) recently reported that NPs-mediated targeting of MAPK pathway can optimize cancer chemotherapy. Polylactic acid glycolic acid (PLGA) NPs conjugated with a selective MAPK inhibitor (PD98059) were taken up by melanoma and lung carcinoma cells and demonstrated a sustained release of the inhibitor resulting in the inhibition of MAPK phosphorylation, inhibition of proliferation and induction of apoptosis. These results were confirmed in vivo on melanoma-bearing mice where the tumour was reduced after this treatment with an enhancement of chemotherapy (Basu et al. 2009). Moreover, the interactions between coated NPs and membrane receptors could lead to various cellular responses depending on the specificity of the receptors.

The role of epidermal growth factor receptor (EGFR) and its ligands was pointed out in several studies. It was clearly demonstrated that in response to DEP and PM 2.5, amphiregulin (AR), a ligand of EGFR, is strongly up-regulated and secreted mostly at the basal side of human airway epithelial cells (Blanchet et al. 2004; Auger et al. 2006). This AR secretion is mediated through the activation of EGFR and ERK MAP kinase pathways and leads to cytokine secretion as AR can induce (GM-CSF) mRNA transcription and protein secretion (Rumelhard et al. 2007). The AR secretion was inhibited by the antioxidant N-acetyl cysteine but not by a neutralizing anti-EGFR, suggesting the trans-activation of EGFR by ROS. This result is of great interest to explain autocrine and paracrine effects as well as remodelling in the lung after exposure to fine and ultrafine PM. However, till now it is not demonstrated for engineered NPs. Interestingly, the group of K. Unfried (IUF, Düsseldorf) has recently demonstrated that CB NPs induce apoptosis and proliferation in rat lung epithelial cells via specific signalling pathways both using EGFR (Sydlik et al. 2006). Both endpoints are induced independently by specific signalling pathways depending on the activation of EGFR. But NP-induced proliferation alone was dependent on the β1 integrins and ERK 1–2 activations, whereas the induction of apoptosis was correlated to c-Jun kinases phosphorylation (Fig. 3). Moller et al. (2005) showed that CB NPs impair phagosome transport and cause cytoskeletal dysfunctions with a transient increase in intracellular calcium. This transient increase in Ca^{2+} was not associated with the induction of ROS since antioxidants did not suppress the response (Moller et al. 2005). This effect of CB NPs could be due to a direct effect on ion channels which control the calcium homeostasis in the cell. Even if all the mechanisms are not completely demonstrated, it appears now clearly that trans-membrane receptors are implicated in NP-induced cell signalling and could lead to specific biological responses to NPs.

In a recent study, Bhabra et al. (2009) showed the abilities of NPs to cause toxicity across a biological barrier. They showed that cobalt–chromium NPs can damage human fibroblast cells across an intact cellular barrier, the BeWO barrier, but without a translocation of NPs. The

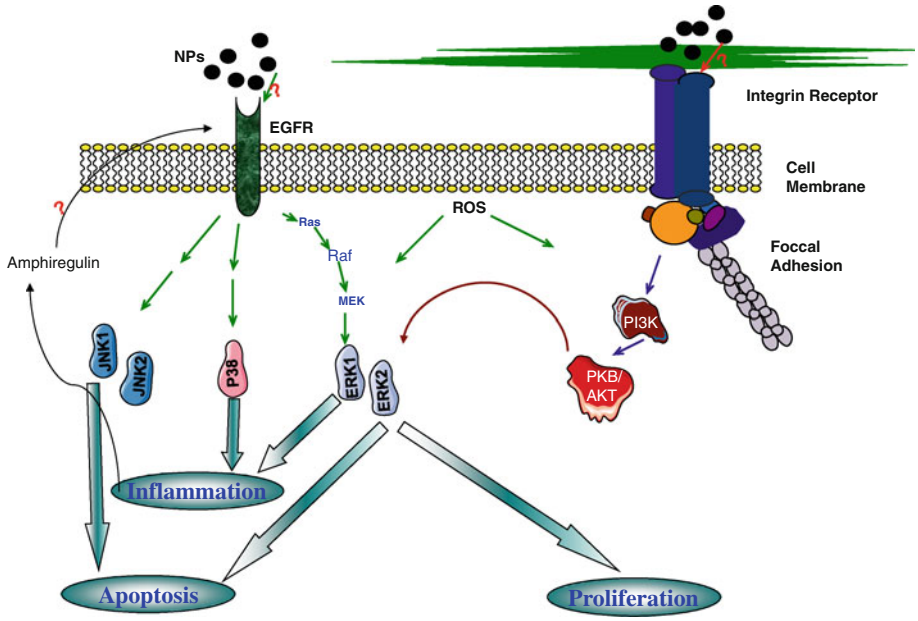

Fig. 3 NP-induced signalling cascades lead to different cellular outcomes. NP-induced activation of EGF receptor can lead to apoptosis, inflammation or proliferation. While activation of integrin receptor and ROS also contribute in proliferation signalling induced by NPs. The role of EGF ligands such as AR needs to be elaborated (Adapted from Unfried et al. 2007)

DNA damage in fibroblasts (measured by comet assays) is mediated by signalling within the barrier through the gap junctions (Bhabra et al. 2009). This signalling is supposed to involve purine nucleotides such as ATP and purine receptors on the fibroblast cell surface. It is the first time that an indirect effect, which could be compared to the radiation-induced bystander effect, is observed with NPs. This area of research is very important, and there is need for further advancements along the same lines.

Nanoparticles and cell death

In addition to adaptive cellular responses, NPs has also been shown to induce cell death in a variety of in vitro systems. Indeed, the effects of NPs depend upon the concentration and duration of exposure. NPs have been shown to induce either apoptotic or necrotic cell death (Pan et al. 2009; 2007; Sydlik et al. 2006). This induction of cell death mechanisms by NPs might act as the basis of different pathologies. As apoptotic processes are involved in different pathological conditions, an understanding of NP-induced apoptosis pathways will assist the development of therapeutic strategies to counteract pathogenesis of such disorders. Some literature reports have described induction of apoptosis after NP exposure (Sydlik et al. 2006; Long et al. 2006; Pan et al. 2007; Vamanu et al. 2008), but these studies did not address the molecular pathways involved in this process. We have recently studied the molecular pathways of apoptosis induction by CB and TiO_2 NPs in human bronchial epithelial cells and have shown that the initial phase of apoptosis induction depends upon the chemical nature of the NPs (Hussain et al. 2010). NPs might exert apoptotic effects through differential signalling events involving death receptor, mitochondria or lysosomes. Lysosomal permeabilization has also been shown to be important in silica NP-induced apoptosis (Thibodeau et al. 2004).

The JNK pathway is comparatively more widely studied in relation to apoptosis induction by NPs. Recently, the involvement of JNK/P38 pathway in the apoptosis induction by TiO_2 NPs has been demonstrated in phytohemagglutinin-stimulated lymphocytes (Kang et al. 2009). In contrast, fullerene NPs have been shown to selectively inhibit JNK-related apoptosis in cerebral microvasculature endothelial cells (Lao et al. 2009).

A thorough understanding of the molecular events induced after NP exposure is crucial as this will allow a better understanding of the biological effects engendered by NPs.

Nanoparticles and cell cycle

In a recent study, Huang et al. (2009a) showed that short-term (24–72 h) and long-term exposure (12 weeks) to non-cytotoxic concentrations of TiO_2 NPs on cultured fibroblast cells (NHI 3T3) could enhance cell proliferation and growth as well as formation of multinuclei, polynuclei and polyploidy (Huang et al. 2009a). They demonstrated for the first time that the cell cycle was modified with a G2/M delay and a slower cell division in long-term exposed cells. The microscopic observation showed multipolar mitotic spindles and aberrant chromosome segregation. Polo-like kinase 1 (PLK1), one of the regulatory proteins implicated in the mitotic phase progression, was deregulated in long-term exposed cells. Normally, PLK1 is translocated from

the cytoplasm to the midzone central spindle and participate in the mitotic progression (Archambault and Glover 2009). Huang et al. (2009a) showed using an anti-PLK1 antibody that Plk1 is located neither in central spindle nor in midzone in the treated cells.

They proposed that deregulation of PLK1 involved by TiO_2 NPs could be the mechanism which leads to chromosome instability, mitotic deregulation and aneuploidy. These preliminary observations need to be improved with further studies and especially in vivo studies since this could explain at least in part TiO_2 NP genotoxicity and tumorigenicity. There is need for further studying these mechanisms with other types of NPs to know whether these could be seen with other types of NPs or these are specific to TiO_2.

Conclusion

In this paper, we have reviewed new data on NP-induced cellular responses. It is true that these new insights give a more complex scenario of the signalling pathways involved in response to various NPs. The paradigm of oxidative stress proposed to explain most of the cytotoxic effects of NPs is not yet given up but is debated because very similar oxidative stress effects in cells cultures induced by different particles (including NPs) could lead in vivo to diverse pathological effects. The increase in ROS in cells after a stress is a common mechanism in toxicology, and it is now obvious that the level of cellular responses to this increase depend on the perturbation of the redox balance. It is striking that whatever the kind of cells and the kind of NP the responses are largely the same with a few number of induced signalling pathways [mostly MAP kinases and redox-sensitive nuclear factors (NFκB, AP1, Nrf2)]. However, it could lead to different biological responses depending on the differentiation of the cell targets which is one of the explanations for the variety of diseases observed after occupational or environmental exposure to well-known particles or fibres.

The interest of the recent researches on the cellular mechanisms induced by NPs is to take into account the specificity of the cells and of their microenvironment. The role of the interactions between the particles, biological fluids and extra cellular matrix appears now to be essential, not only for the uptake but also for the induction of a specific response via the interaction with the membranes, specific receptors or lipid rafts. The large variety of engineered NPs in the market and under development makes these studies very complicated. However, the development of safe nanomaterials depends on better knowledge of these specific interactions. Another interesting finding is the ability of NPs to develop a response without a direct contact with the cells but after an induction of secreted factors. For the first time, it was demonstrated for cytokines and for EGFR ligands which can have autocrine and paracrine effects. Now, it appears that small molecules such as purines could be increased in response to NPs, transferred through the barrier gap junctions and diffuse in a medium to activate specific receptors. These specific responses could explain the in vivo observed differences. The last but not least is the possibility for NPs (after their interactions with proteins, enzymes, cytokines, growth factors, outside or inside the cell) to modify the functions of these proteins with a possible indirect pathological effect.

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