Research Paper

Silver nanoparticles can attenuate nitrative stress

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ABSTRACT

We have reported previously that glucose availability can modify toxicity of silver nanoparticles (AgNPs) via elevation of antioxidant defence triggered by increased mitochondrial generation of reactive oxygen species. In this study, we examined the effect of glucose availability on the production of reactive nitrogen species in HepG2 cells and modification of nitrative stress by AgNPs. We found that lowering the glucose concentration increased expression of genes coding for inducible nitric oxide synthase, NOS2, and NOS2A resulting in enhanced production of nitric oxide. Surprisingly, AgNPs decreased the level of nitric oxide accelerated denitration of proteins nitrated by exogenous peroxynitrite in cells grown in the presence of lowered glucose concentration, apparently due to further induction of protective proteins.

1. Introduction

In recent years interest in silver nanoparticles and their applications has increased mainly because of the important antimicrobial activities of these nanomaterials, allowing their use in several industrial sectors. These broad applications, however, increase human exposure and thus the potential risk related to their short- and long-term toxicity. There is increasing concern with respect to the biological impacts of the use of silver nanoparticles on a large scale, and the possible risks to the health and environment [1]. A large number of in vitro studies indicate that AgNPs are toxic to the mammalian cells derived from skin, liver, lung, brain, vascular system and reproductive organs. Interestingly, some studies have shown that these particles have the potential to induce genes associated with cell cycle progression, DNA damage and apoptosis in human cells even at non-cytotoxic doses [2].

An important mode of action of AgNPs is the induction of oxidative stress. Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses or, more recently, as disruption of redox signaling and control [3]. Numerous data exist in the literature that both ROS and reactive nitrogen species (RNS) are produced in a well-regulated manner to help maintain homeostasis at the cellular level in the normal healthy tissues, play an important role as second messengers, and control the activities of several enzymes, first of all guanyl cyclase [9]. At the cellular level, NO affects cell apoptosis and participates in immune defense [10] while at the organismal level its main function is the control of blood pressure [11].

While NO shows a limited reactivity, a product of its reaction with the superoxide radical anion, peroxynitrite ONOO⁻, is a reactive oxidant and nitrating agent. The reaction of peroxynitrite formation is characterized by a very high rate constant, of about 1.6*10¹⁰ [12] so it proceeds in vitro even though the concentrations of both substrates are low and is enhanced when production of the precursors is increased or when the activity of superoxide dismutase is compromised (which occurs in many pathologies). Peroxynitrite is unstable under physiological conditions, one way of its decomposition leading to the formation of the hydroxyl radical and nitrogen dioxide [13]. It is therefore a strong oxidant and nitrating agent modifying proteins, lipids and nucleic acids and depleting cellular antioxidants. Proteins are subject to various modifications by peroxynitrite, the most typical modification being nitration of tyrosine residues [14,15].

Oxidative stress induced by engineered NP is due to acellular factors such as particle surface, size, composition, and presence of metals, while cellular responses include mitochondrial respiration, NP-cell interaction, and immune cell activation are responsible for ROS-
mediated damage. NP-induced oxidative stress responses are torch bearers for further pathophysiological effects including genotoxicity, inflammation, and fibrosion as demonstrated by activation of associated cell signaling pathways [16]. Various factor, among them glucose availability, modulate AgNPs-induced oxidative stress [17] or the presence of other xenobiotics. The main cellular source of superoxide is the respiratory chain in the mitochondria [18]. If oxidative stress caused by increased activity of the respiratory chain is of moderate intensity, the cell can adapt to its occurrence [17], which results in an increased resistance to oxidative stress induced by other agents, e.g. AgNPs. Adaptation is mainly due to activation of the Nrf2 pathway [19], NF-kB [20] or the MAPK pathway [21]. Metabolic pathways regulated by oxidative stress control the level of many proteins responsible for the cellular redox balance.

In this paper, we have studied how AgNPs can modulate nitrative stress induced by increased production of reactive oxygen species in mitochondria and increased activity of iNOS in HepG2 cells.

2. Materials and methods

2.1. Cell culture

HepG2 cell line was derived from hepatocellular carcinoma of a Caucasian male adolescent. HepG2 cells can be cultured in media containing different concentrations of glucose [22] and are a well recognized model for nanotoxicity testing. HepG2 cell line originated from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with FBS (Gibco). Cells were maintained in a 5% CO2 atmosphere at 37 °C, at 95% relative humidity. During passages cells were detached from culture surface with trypsin solution (0.25 trypsin, 1 mM EDTA in PBS, 10 min incubation in culture conditions).

2.2. Neutral red viability assay

Cells were plated on 96-well plates (Nunclon) at a density of 15,000 cells per well in a final volume of 0.1 cm3. After 24 h, an appropriate aliquot of AgNPs was added to achieve concentrations in range from 2.5 to 50 µg/cm3 in a final volume of 0.2 cm3. The procedure of preparation of nanoparticles is as described by Zuberek at al. [17]. After next 24 h incubation, the medium was removed and cells were washed twice with 0.15 cm3 per well of PBS solution. The cells were then flooded with 0.1 cm3 of neutral red solution (50 µg/cm3 neutral red in the culture medium). After a 4h incubation at 37 °C in an atmosphere of 5% CO2 neutral red solution was discarded, the cells washed twice with 0.15 cm3 PBS and 0.15 cm3 of fixative (50% ethanol, 49% H2O, 1% acetic acid) was added to each well. The plate was shaken for 15 min and the absorbance was measured at a wavelength of 540 nm using an EnVision™ Multilabel Reader (Perkin-Elmer) [23].

2.3. Gene expression analysis

Total RNA was isolated from 106 HepG2 cells employing MagNA Pure LC 2.0 Instrument (Roche) according to the manufacturer’s protocol. Genomic DNA was removed by DNase I digestion (RNase free DNase, Life Technologies) and 1 µg of the total RNA was reverse-transcribed using the SuperScript™ III First-Strand Synthesis SuperMix (Life Technologies). qPCR analysis was performed with C1000 Thermal Cycler–CFX96 Real-Time System (BioRad). Primers were obtained from RealTimePrimers.

2.4. Nitric oxide measurement

Cells for flow cytometry evaluation of nitric oxide were plated on 6-well plates in density of 167·105 cells/cm3 in 3 cm3 of low or high glucose DMEM. AgNPs were added after 24 h to final concentration of 25 µg/ml for another 24, 48 or 72 h of incubation, after which cells were trypsinized and resuspended in 1 cm3 of culture medium. DAF-FM was added to final concentration of 5 µM and fluorescence intensity was recorded in FITC channel (LSRII flow cytometer) after 30 min of incubation in 37 °C.

2.5. Measurement of reactive oxygen species

Cells for flow cytometry evaluation of nitric oxide were plated on 6-well plates in density of 167·105 cells/cm3 in 3 cm3 of low or high glucose DMEM. AgNPs were added after 24 h to final concentration of 25 µg/ml for another 4 h of incubation, after which cells were trypsinized and resuspended in 500 mm3 of full growth media. After 10 min in 37 °C 100 mm3 of DMEM solution of appropriate probe was added to cell suspension to form final concentration of 5 µM for another 45 min of incubation. Cellular levels of hydrogen peroxide, cellular superoxide anion and mitochondrial superoxide anion was measured respectively with DHR123, DHE and MitoSOX.

2.6. Measurement of nitrotyrosine in HepG2 cells

Cell cultures grown in 6-well nuncion delta plates at density of 150,000 cells/cm3 were incubated for 5 min with peroxynitrite in concentration of 1 mmol/dm3 after 2 h of incubation with AgNPs (25 µg/ml), after peroxynitrite treatment monolayer was washed twice with PBS. Cells were lysed and scrapped in RIPA buffer immediately after peroxynitrite incubation and after another 2 or 4 h of incubation in culture conditions.

2.7. Measurement of nitrotyrosine

Tyrosine in concentration of 1 mmol/dm3 was nitrated with 1.4 mmol/dm3 peroxynitrite in presence of AgNPs in concentration of 12.5 µg/cm3 or in the presence of BSA in concentration corresponding to one used in preparation of AgNPs suspension. Fluorescent spectra of tyrosine were obtained at 315 nm excitation wavelength.

3. Results

3.1. Cell survival

Comparison of the effects of AgNPs on cell survival showed that cells grown in 25 mM glucose and transferred to medium of a lower glucose concentration (5.5 mM) for 48 h were more resistant than cells maintained in 25 mM glucose (Fig. 1). This result confirms our previous observations on the higher toxicity of AgNPS to HepG2 cells grown on high glucose as compared with cells cultivated on low glucose for a prolonged time [17].

![Fig. 1. Effect of AgNPs treatment on the survival of HepG2. Cells were grown in high glucose DMEM and transferred to either high glucose DMEM (25 mmol/dm3) or low glucose DMEM (5.5 mmol/dm3). After 48 h, cells were treated with AgNPs (2.5–50 µg/cm3). After 24 h of incubation viability was assessed with neutral red method. Curves were compared with extra sum of square F test and are statistically different, p=0.0118, N=12 (high glucose), N=9 (low glucose).](image)
3.2. Analysis of gene expression

Lowering glucose concentration in the culture medium increased the mRNA level for NOS2 and NOS2A. No significant changes were found for NADPH oxidase 5, the main extramitochondrial source of superoxide radical (Table 1).

3.3. Nitric oxide level

Decrease of glucose concentration from 25 down to 5.5 mol/dm³ lead to stabilization of intracellular nitric oxide concentration at higher level after 72 h but not at 24 or 48 h (Fig. 2). This level was not different from that found in cells grown on low-glucose medium for a month or longer. These results demonstrate that a new redox balance is attained in the cells after 3 days, apparently being an element of cellular adaptation to augmented oxidative stress caused by lowered glucose concentration.

Comparison of nitric oxide levels in cells grown on different glucose concentrations revealed that AgNPs did not induce any detectable increase in nitric oxide in cells grown in 25 mM glucose and even decreased nitric oxide generation in cells grown in 5.5 mol/dm³ glucose (Fig. 3).

3.4. Production of reactive oxygen species

Cystosolic superoxide level was considerably lower in cells grown in high glucose than in cells grown in low glucose. AgNPs did not affect significantly the cystosolic superoxide level in cells grown in both high and low glucose concentrations (Fig. 4A). Mitochondrial superoxide level measured with MitoSOX was also lower in cells grown in low glucose (as compared with cells grown in high glucose); AgNPs increased superoxide level in cells grown in high glucose while not affecting superoxide production in cells grown in low glucose (Fig. 4C). The cellular level of peroxides reflected that of mitochondrial superoxide (Fig. 4B), confirming that mitochondria were the main source of cellular peroxides in HepG2 cells.

3.5. Nitrotyrosine level

Endogenous level of protein nitration in HepG2 cells was below the detection level of the ELISA test used. Protein nitration induced by exogenous peroxynitrite was higher in cells grown in low-glucose medium. AgNPs treatment tended to increase the level of protein nitration in cells grown on high glucose (not reaching statistical significance) and did not affect the level of protein nitration in cells grown on low glucose. The level of nitrotyrosine decreased during subsequent incubation, the rate of denitration being lower in cells grown in 5.5 mM glucose. After 2–4 h the level of nitrotyrosine dropped down below the detection limit. In cells grown on low glucose, the rate of nitrotyrosine removal was higher in the presence of AgNPs (Fig. 5).

Control experiments in which tyrosine nitration was determined fluorimetrically demonstrated the AgNPs inhibited nitration of tyrosine (Fig. 6).

4. Discussion

Our previous paper demonstrated that glucose availability can modulate the toxic action of AgNPs via regulation of levels of proteins involved in cellular antioxidant defense [17].

We have found an increased activity of mitochondria, responsible for oxidative phosphorylation, under lowered glucose conditions. This effect activates signaling pathways controlled by cellular levels of ROS. We expected that stress induced by lowered glucose will be augmented by stress induced by nanoparticles. To the contrary, the cells cultured after prolonged (over one month) under low glucose conditions were found to be less susceptible to AgNPs than cells grown in 25 mM glucose. We ascribe this effect to the stimulation of antioxidant defense in cells stressed by low glucose conditions, as evidenced, i. a., by increased activities of main antioxidant enzymes [17]. This process of adaptation requires time and first discernible effects can be observed at 48 h after the change of the medium.

Induction of oxidative stress induced by transfer of the cells to increased glucose concentration has been reported in the literature. This effect is ascribed to the production of free radicals in the process of glucose autoxidation. Glucose can enolize and form ROS (superoxide anion and hydrogen peroxide) even under physiological conditions [24]. Increased generation of ROS may slow down the rate of cell proliferation [25]. However, some authors reported induction of ROS generation by cell transfer to medium containing low glucose concentration. This effect took place already 15–30 min after the lowering of glucose concentration in the medium and was inhibited by rotenone, which points to Complex I of the respiratory chain as the main source of ROS [26].
In this paper, we used HepG2 as a model cell line. This line adapts easily to various culture conditions [22]. ATCC recommends culturing these cells in medium containing 1 g/L of glucose but these cells grow well in a medium containing 25 mM glucose, and only 50 mM glucose limits cell survival and induces apoptosis after prolonged incubation (72 h) [27]. HepG2 cells are derived from hepatocytes, in which glucose conditions may be high due to glycogenolysis and gluconeogenesis as these cells produce glucose and release it to the bloodstream so high glucose conditions may imitate better the in vivo conditions for these cells [28].

Analysis of survival data and expression of oxidative stress-related genes [17] allows to conclude that adaptation of HepG2 cells to new culture conditions requires at least 72 h. A significant increase in the level of transcript coding inducible NOS has been observed not earlier but after 72 h of culture in low glucose medium. This enzyme is responsible for generation of relatively high concentrations of nitric oxide in the cells. The increased activity of iNOS is maintained during at least one-month culture of the cells in low glucose (not shown). Apparently, this stimulation of iNOS expression may be due to disturbances in the redox balance caused by increased activity of the mitochondrial respiratory chain.

Transcriptional regulation of the gene responsible for the production of iNOS is complex and still not fully understood. Several signaling cascades have been found to control the expression of iNOS in cells at the transcriptional level, including JNK/ p38, NF-κB, JAK and STAT [29]. The iNOS promotors are controlled by numerous transcription factors, the most important being NF-κB p50: p65. This factor is activated, i. a., by lipopolysaccharide (LPS), viral products, cytokines, cell–cell contact, neurotoxins and oxidative stress [6]. The C/EBP transcription factor, activated by LPS, viral products, CD40 ligation, cAMP, hypoxia IL-1β, dsRNA, glucose metabolites acts probably as a co-activator [30]. The STAT-1 transcription factor has been identified.
as a negative regulator of the iNOS gene [31,32]. The role of AP1 proteins in the regulation of iNOS promoter is still controversial; it was found, however, that Fra-1, Fra-2, JunB, JunD, and FosB transcription factors are involved in the regulation of iNOS expression and NO production [33,34]. On this basis it can be expected that ROS play a key role in the regulation of iNOS expression and a disturbance in the redox balance may lead to an increased NO generation in the cells.

We observed decreased levels of cytoplasmic superoxide and hydrogen peroxide in cells grown on low glucose upon incubation with AgNPs (Fig. 4). Increased ROS generation by nanoparticles has been reported by many authors. However, increased generation of hydrogen peroxide in cells treated with AgNPs is usually found in high-glucose media (or precise information on culture conditions is lacking) and the studies do not consider the endogenous ROS production by mitochondria and its effect on the redox state of the cells.

This effect may not reflect the true level of superoxide but to be due to the lower accessibility of superoxide for the fluorogenic probe. Superoxide reacts rapidly with NO forming peroxynitrite. This reaction proceeds at a very high rate and is diffusion-controlled \((k \approx 1 \times 10^{10} \text{ M}^{-1} \text{s}^{-1})\). The reaction rate constant of superoxide with dihydroethidine is much lower \((2.17 \pm 0.059 \times 10^6 \text{ M}^{-1} \text{s}^{-1})\), so in spite of the higher concentration of the probe with respect to NO, the latter may compete significantly for superoxide, the more that it is constantly produced in the system [35]. The mitochondrial production of superoxide was increased in cells grown on low glucose (Fig. 4), which may underlie this effect. Similarly, the level of NO was decreased in cells grown in low glucose by treatment of AgNPs. Both these observations are compatible with the hypothesis that increased consumption of superoxide and NO can explain the lower level of superoxide, hydrogen peroxide and NO found in cells grown on low glucose, inducing higher iNOS expression. Alternatively, it can be suggested that AgNPs increase biosynthesis of antioxidant proteins which decompose ROS and inhibit the activity or attenuate the expression of iNOS, especially under low glucose conditions.

We found increased protein nitration in cells grown on low glucose by exogenous peroxynitrite. Nitrotyrosine formation is one of the main protein modifications induced by peroxynitrite. The level of protein nitration by endogenously produced peroxynitrite is low due to the efficient protection against peroxynitrite and protein denitratase activity. Indeed, low levels of peroxynitrite could be detoxified by enzymatic and nonenzymatic systems [36–39]. In our experiments, no increased protein nitration by endogenously formed peroxynitrite was found in cells grown on low glucose. This results are apparently conditioned by low yield of nitration under physiological and cell culture conditions. It has been reported that even under inflammatory conditions, one to five 3-nitrotyrosine residues per 10,000 tyrosine residues are detected [40,41], due mainly to repair mechanisms of the tyrosyl radical such as reduction by glutathione and to metabolism of 3-nitrotyrosine.

The small fraction of nitrated protein under physiological conditions cast doubt on its possible biological relevance. It is noteworthy to indicate, however, that a relatively limited number of proteins are preferential targets of nitration, and within these proteins, only one or a few specific tyrosines can be nitrated.

Addition of a \(-\mathrm{NO}_2\) group to tyrosine lowers the \(pK_a\) of its phenolic hydroxyl group by 2–3 units and adds a bulky substituent; if placed on relevant tyrosines, nitration can alter protein function and conformation, impose steric restrictions, and also inhibit tyrosine phosphorylation. However, to have biological significance, a loss-of-function modification requires a large fraction of protein to become nitrated at specific critical tyrosines and result in 3-nitrotyrosine to tyrosine ratios in the range of 0.1–1.0; documentation of these large ratios for a given protein in vivo is scanty, and it is doubtful that many proteins will undergo such an extent of nitration.

Our study demonstrates effective removal of nitrotyrosine from cellular proteins nitrated by exogenous peroxynitrite (Fig. 5). In the work of Aghdam et al. [42] it was shown that activity of proteasome is directly connected with glucose levels in intracellular environment; proteasome activity is elevated at higher glucose concentration. Our research shows that glucose concentration in culture media influences the rate of removal of modified proteins. Higher concentration of glucose promotes faster degradation of nitrated proteins. AgNPs increase the rate of degradation at lower glucose concentration due to oxidative stress [43] activating NRF2 signaling pathway, which in turn elevates proteasome activity [44].

The presence of a nitrosyrosine denitrase activity, independent of digestion of nitrated proteins, was first reported by Kamisaki et al. [45]. According to the present knowledge, denitrase activity can be described as an oxygen-regulated and endotoxin-induced tissue-specific process, detectable in brain, spleen, and lung of LPS-treated rats, in untreated rat heart and brain as well as in LPS-treated RAW 264.7 cells [46]. The present strongly suggest that the denitrase activity is present also in HepG2 cells and is higher in cells grown on high glucose. However, since we did not use protease inhibitors, it cannot be excluded that the removal of nitrotyrosine residues is partly dependent on proteolysis of nitrated proteins.

The in vitro test (Fig. 6) demonstrated that the presence of silver nanoparticles attenuates nitrotyrosine formation, apparently due to peroxynitrite decomposition. However, in the cellular system the presence of AgNPs did not protect cellular proteins against nitration, in cells grown on low or high glucose. Apparently, more efficient peroxynitrite-decomposing mechanisms present in the cells make the role of AgNPs marginal in cellular systems, in contrast to the simple system of peroxynitrite + tyrosine. The increased de-nitration of proteins in cells grown on low glucose incubated with AgNPs is noteworthy. There are no data on denitrase activity of AgNPs and such a mechanism does not seem probable. Rather, AgNPs can be expected to induce synthesis of defensive proteins which may include those showing denitrase activity or proteolytic activity.

Lipid peroxidation can be described as an oxidation of lipids containing double carbon-carbon bonds leading to formation of peroxides. Most susceptible to peroxidation are polyunsaturated fatty acids (PUFAs), but other lipids like glycolipids, phospholipids (PLs) and cholesterol can undergo the process as well. Lipids can be oxidized in enzymatic processes, involving lipoxygenases, cylooxygenases and cytochrome P450 and non-enzymatically in a chain process initiated by free radicals.

Lipid peroxidation was initially studied as a deleterious outcome of oxidative stress, however the regulatory aspect of the process was also noticed [47]. Lipid peroxidation is a physiological process proceeding under physiological conditions but is enhanced in oxidative stress. Under conditions of mild oxidative stress, lipid peroxidation products upregulate antioxidant protection system which in turn limits the harmful effects of oxidative stress. However intensified lipid peroxidation can lead to cell death by apoptosis, necrosis or autophagy [48–50]. Most extensively studied secondary products of lipid peroxidation are malondialdehyde (MDA), propanal, hexanal and 4-hydroxynonenal (4-HNE). MDA and 4-HNE were described respectively as most mutagenic and most toxic products of lipid peroxidation [51]. 4-HNE is considered as one of the major lipid peroxidation products and mediating secondary effects of oxidative stress [52]. High toxicity of 4-HNE is tied with its high reactivity against thiol and amino groups [53]. It was found that 4-HNE upregulates many transcription factors e.g. nuclear factor erythroid 2-related factor 2 (Nrf2) [54–56], activating protein-1 (AP-1) [57], NF-kB [58] and peroxisome-proliferator-activated receptors (PPAR) [59,60], mitogen-activated protein kinases (MAPK) and other stress response pathways [61]. Non-physiological levels of 4-HNE can promote development and progression of pathological states such as neurodegenerative diseases [62], diabetes mellitus [63], and carcino genesis [64,65]. In our previous work [17] we have shown that change in environment glucose level promotes cell adaptation to oxidative stress. 4-HNE can be one of the key signaling molecules behind this adaptation process, the more that nanoparticles have been
demonstrated to promote lipid peroxidation [66].

In summary, AgNPs do not induce nitrative stress in HepG2 cells but even inhibit nitration in a cell-free system, most probably by reacting with peroxynitrite (although this effect seems to be of no importance in cellular systems) and accelerate the de-nitration of tyrosine residues. Apparently, the action of AgNPs in HepG2 cells is not direct but mediated by induction of biosynthesis of defensive proteins, especially in cells grown in low glucose. This question requires further studies.

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