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Intracellular trafficking pathways in silver nanoparticle uptake and toxicity in *Caenorhabditis elegans*

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Nanotoxicology; Nanoparticle uptake; *In vivo* endocytosis; Lysosome

Introduction

The volume of production and breadth of applications of engineered nanoparticles (NPs) allow for significant exposure to humans [Reviewed by (Elsaesser and Howard, 2012)]. NPs are internalized into cells and subsequently sorted into lysosomes, as observed in studies using functionalized polystyrene NP uptake in immune cells, acidic poly (DL-lactide-co-glycolide; PLGA) and poly (DL-lactide) NPs in an epithelial cell line, and with iron oxide, silica, titanium dioxide, and PLGA NPs in brain-derived endothelial cells (Lunov et al., 2011, Baltazar et al., 2012, Kenzaoui et al., 2012). Recent studies suggest that endocytosis is an important mechanism for intracellular NP uptake (Iversen et al., 2011, Zhang et al., 2009, Vacha et al., 2011, Kim and Choi, 2012), and NPs have been detected in early endosomes, late endosomes, and lysosomes (Zhang and Monteiro-Riviere, 2009, Kenzaoui et al., 2012).

Exposure to silver nanoparticles (AgNPs) is of particular concern, as they are comparatively more toxic than other metal nanoparticles (Hussain et al., 2005), and are incorporated into various consumer products as an antibacterial component (Lem et al., 2012). AgNP uptake and associated toxicity have been extensively studied both *in vitro* and *in vivo* (Hafeli et al., 2009, Hauck et al., 2008, Lenaerts et al., 1984, Miao et al., 2010, Yang et al., 2012, Shoults-Wilson et al., 2011, Gorth et al., 2011). Intracellular uptake of AgNPs is important to their toxicity (Miao et al., 2010, Meyer et al., 2010). A body of *in vitro* studies shows that endocytosis is a major mechanism underlying the cellular uptake of AgNPs (Wang et al.,

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Declaration of interest

The authors report no conflicts of interest in this work.

2012, Kim and Choi, 2012, Gao et al., 2013, Gliga et al., 2014), which is directly associated with cytotoxicity (Caballero-Diaz et al., 2013). There is also evidence for AgNP uptake and toxicity in prokaryotic cells (Ivask et al., 2014). *In vitro* studies of AgNP uptake demonstrate that their toxicity is dependent on both clathrin-mediated endocytosis and micropinocytosis (AshaRani et al., 2009). However, *in vivo* investigations of endocytosis in AgNP uptake have been limited (Khan et al., 2015), and the majority of uptake studies have focused on biodistribution (mostly through visualization and quantification) (Dziendzikowska et al., 2012) rather than on toxic endpoints. We investigated specific components of endocytic mechanisms involved in AgNP toxicity *in vivo* through the use of pharmacologic inhibitors of endocytosis and endocytosis-deficient mutants in *C. elegans* (*rme-1*, *rme-6*, *rme-8*). RME-1 is necessary for endocytic recycling (Grant et al., 2001, Lin et al., 2001), RME-6 is a regulator of early endosome formation (Sato et al., 2005), and RME-8 is necessary for pre-lysosomal sorting of endosomes (Zhang et al., 2001).

Lysosomes are an important sink for NPs and exposure to several types of NPs results in alterations in lysosomes including lysosomal membrane integrity, lysosomal pH, and activity of lysosomal sulfatases (Frohlich et al., 2012, Baltazar et al., 2012, Caballero-Diaz et al., 2013). In *C. elegans*, *cup-5* mutants have excess lysosomes (Hersh et al., 2002) and defective proteolytic degradation in autolysosomes, leading to inefficient lysosomal regeneration (Sun et al., 2011). CUP-5 protein localizes to lysosomes (Campbell and Fares, 2010), while GLO-1 colocalizes with lysosome-related gut granules (intestine-specific lysosome-related sites) (McGhee, 2007). GLO-1 protein is associated with lysosomal biogenesis (Artal-Sanz et al., 2006) and gut granule formation (Rabbitts et al., 2008). *glo-1* mutants lack autofluorescent gut granules and the acidified endocytic compartment (Hermann et al., 2005). We previously found that most of the toxicity of several Ag NPs could be attributed to dissolved Ag release from the nanoparticles (Yang et al., 2012). The exception to this result was the citrate-coated AgNPs, which were the least soluble of the test group and caused significant toxicity via mechanisms other than dissolution (Yang et al., 2012). Therefore, we hypothesized that nanoparticle-specific pathways such as endocytosis would be critical to citrate-coated AgNP uptake and exposure. We used both pharmacological endocytosis inhibitors and endocytosis- and lysosome-related mutants to investigate the roles of endocytosis and lysosomes in regulating *in vivo* responses to AgNPs. Our results support the hypothesis that endocytosis is important for AgNP uptake in *C. elegans*, and provides mechanistic evidence for early endosome formation in clathrin-mediated endocytosis as a necessary component of AgNP toxicity *in vivo*.

Materials and Methods

C. elegans culture conditions

C. elegans were cultured in petri dishes on K-agar seeded with OP50 strain *Escherichia coli* (Williams and Dusenbery, 1988) to prepare the nematodes for liquid medium exposure, which was carried out in 96-well plates as previously described (Meyer et al., 2010) except as detailed below. Strains N2 (wild-type Bristol), DH1201 (*rme-1* deletion, endocytosis defects in oocytes and coelomocytes, outcrossed 2 times), DH1370 (*rme-6*, point mutation, decreased or absent coelomocyte-mediated endocytosis, outcrossed 3 times), DH1206

(*rme-8*, point mutation, endocytosis defects in oocytes and coelomocytes, outcrossed 3 times), GS2477 (*cup-5*, point mutation, defective in endocytosis by coelomocytes and lysosomal degradation, outcrossed 3 times), JJ1271 (*glo-1*, point mutation, lysosome-related gut granule formation deficient, not outcrossed) were obtained from the *Caenorhabditis* Genetics Center (CGC; Minneapolis, MN, USA).

Nanoparticles and chemicals

The citrate-functionalized AgNPs (herein referred to as CIT-AgNPs) had a size distribution of $25 \text{ nm} \pm 9 \text{ nm}$ and have been previously characterized (Yang et al., 2014). Other AgNPs with polyvinylpyrrolidone (PVP) and gum arabic (GA) coatings described previously (Yang et al., 2012). Experiments performed in this study used the same media and temperature as were used in the publications that describe AgNP characterization. Dissolved Ag exposures were performed with a dissolved AgNO_3 stock solution. Chlorpromazine and phenothiazine were purchased from Sigma Aldrich Co. (St. Louis, USA).

24 Hour Lethality Test

Young adult nematodes (age-synchronized by 46 h of growth on OP50 plates at 20°C after egg isolation and overnight hatch without food) (Lewis, 1995) were exposed to AgNPs. Nematodes were dispensed into 96-well plates using a COPAS Biosort with EPA moderately hard reconstituted water (“EPA water” hereafter) as the exposure medium as described (Yang et al., 2012). No bacterial food was added to the dosing medium. After 24 h, nematodes were examined blindly for lethality as described (Yang et al., 2014).

Growth Assay

The growth assay was adapted from Boyd et. al (Boyd et al., 2012). Briefly, synchronized L1 *C. elegans* were dispensed into 96-well plates (50 nematodes per well), exposed to nanoparticles for 48 h (exposure medium described by Yang et al. (Yang et al., 2012)), and growth was assessed by measuring worm size using a COPAS BioSort. Wells were supplemented with UVC-killed UvrA bacteria (rationale described in (Meyer et al., 2010)) every 24 hours to ensure that the nematodes did not starve.

Total silver measurements

For nematode silver content measurement, the exposed nematodes were transferred to clean EPA water with food to allow the gut to clear for 2 h, centrifuged at 2200 rpm for 2 min, rinsed with EPA water 3 times, and freeze-dried (Labconco, US) for 2 d. The freeze-dried samples were digested with concentrated nitric acid (70%, Fisher Scientific, US) at $\sim 90^\circ\text{C}$ for 4 h. The digest was diluted with MilliQ-filtered water ($>18 \text{ M}\Omega\text{-cm}$, EMD Millipore), held overnight at room temperature, and further diluted with mixed acid (2% HNO_3 and 0.5% HCl v/v) prior to elemental analysis by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700x).

Statistical analysis

We used Microsoft Excel to carry out data plotting and R (SAS institute) for Kruskal-Wallis tests and analysis of variance (ANOVA). Processing of COPAS output (Time of Flight and Extinction analysis) was as previously described (Yang et al., 2012).

Results

Pharmacological inhibition of endocytosis reduced AgNP but not AgNO₃ toxicity

Chlorpromazine is an inhibitor of clathrin-mediated endocytosis (Rejman et al., 2005). At the doses we used and up to 40 mg L⁻¹, chlorpromazine alone did not cause observable toxicity (data not shown). Chlorpromazine did not significantly alter AgNO₃ toxicity, but almost eliminated CIT-AgNP-induced toxicity (Fig. 1). We attempted similar experiments with phenothiazine, another inhibitor of clathrin-mediated endocytosis. However, after mixing phenothiazine into the dosing solutions, aggregates formed, possibly confounding our ability to interpret the rescue effect that we observed in both AgNO₃ (lower concentrations) and CIT-AgNP exposures (Fig. S1).

Genetic deficiency in early endosome formation reduced AgNP uptake and toxicity

To further understand the importance of endocytosis-related pathways in AgNP uptake and associated toxicity, we used three endocytosis-deficient mutants (*rme-1*, *rme-6*, and *rme-8*) to complement the pharmacological inhibitor results. The roles of these and other mutants we used are presented schematically in Fig. 2A.

There were no significant differences in mortality among strains (*rme-1*, *rme-6*, *rme-8*, N2) due to AgNO₃ exposure (one-way ANOVA, $p = 0.57$, Fig. 2B). However, the *rme-1* and *rme-6* strains were more and less sensitive than N2 to CIT-AgNPs, respectively (Kruskal-Wallis test, $p = 0.021$ and $p = 0.008$ for *rme-1* and *rme-6*, respectively). CIT-AgNP-induced lethality in *rme-8* mutants was not significantly different than in wild-type (Kruskal-Wallis test, $p = 0.13$) (Fig. 2C). We detected statistically indistinguishable amounts of total silver in all strains of AgNO₃-exposed nematodes, consistent with the mortality results. However, in CIT-AgNP-exposed nematodes, we found increased and decreased total silver uptake in *rme-1* and *rme-6* respectively, but no significant difference in *rme-8* compared to N2 (Fig. 2D). We found a positive correlation between total silver uptake and CIT-AgNP toxicity ($R^2 = 0.74$) (Fig. 2E). In addition, *rme-6* also showed increased resistance to CIT-AgNPs based on growth inhibition data (Fig. S2B). As a further test of the importance of early endocytosis in the toxicity specific to a poorly soluble AgNP, we compared the toxicity of four additional AgNPs that we previously characterized as significantly more soluble than CIT-AgNPs (Yang et al., 2012), and found that *rme-6* mutants were less sensitive only to CIT-AgNPs compared to wild-type (Table 1).

Acute toxicity of Ag NP and AgNO₃ was unaltered in lysosomal function-related mutants

Neither *cup-5* nor *glo-1* were significantly more or less sensitive than N2 to the lethal effects of AgNO₃ or CIT-AgNPs (Fig. 3A, B) (Kruskal-Wallis test, $p = 0.23$).

AgNP-induced growth inhibition in lysosome mutants

cup-5 and *glo-1* mutants showed more growth inhibition than wild-type upon both AgNO₃ and CIT-AgNP exposure (Fig. S3), suggesting that normal lysosomal function was important for nematode growth both in the context of dissolved silver-related stress conditions, and AgNP-induced stress.

Discussion

The rescue by chlorpromazine of CIT-AgNP but not AgNO₃ toxicity (Fig. 1), in combination with our previous results demonstrating that CIT-AgNPs caused NP-specific toxicity (Yang et al., 2012), supports the hypothesis that CIT-AgNPs are taken up by endocytosis *in vivo*.

More specifically, *rme-6* mutants were more resistant to CIT-AgNP toxicity and accumulated less total silver than wild-type, supporting a mechanistic role for early endosome formation during clathrin-mediated endocytosis in CIT-AgNP toxicity. Others have reported that another endocytosis mutant strain, *rme-2*, was more resistant to gold nanoparticle toxicity than wild type (Tsyusko et al., 2012). However, due to the severe growth retardation of *rme-2* compared to N2 (unpublished observations), we chose not to use this mutant strain. The increased uptake and toxicity in *rme-1* mutants may result from a role for *rme-1* in CIT-AgNP detoxification pathways. Alternatively, it could be caused by a generally compromised physiological state that resulted in greater uptake and sensitivity to CIT-AgNPs, as RME-1 function is necessary for endosomal recycling in *C. elegans* (Grant et al., 2001).

We had hypothesized AgNP toxicity was mediated in significant part by dissolution within acidic lysosomes, and that therefore defective lysosomal function would result in protection against CIT-AgNP but not AgNO₃ toxicity. While no differences in lethality were observed in lysosomal mutants (*cup-5* and *glo-1*) compared to wild-type (Fig. 3), they were more sensitive, rather than more resistant, to growth inhibition by both CIT-AgNPs and AgNO₃ compared to N2 (Fig. S3). These observations are not inconsistent with the possibility that lysosomal-associated dissociation of AgNPs could be a contributor to the observed toxicity (the “Trojan Horse effect”) (Sabella et al., 2014), but based on this hypothesis it is not clear why these mutants would also be more sensitive to AgNO₃. The sensitivity of these lysosomal function mutants to AgNO₃ is also consistent with another hypothesis: that lysosomal deficiency results in sensitivity to multiple stressors, potentially due to a reduced ability to recycle damaged macromolecules.

In summary, we provide mechanistic evidence that uptake and toxicity of CIT-AgNPs were dependent on early endosome formation during clathrin-mediated endocytosis in *C. elegans*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Dose response curves for ionic silver and AgNP toxicity with and without chlorpromazine (10 mg L^{-1}). A) AgNO_3 (0.025–0.1 mg-Ag L^{-1}); B) CIT-AgNPs (0.1–1.5 mg-Ag L^{-1}).

Figure 2.

A) Schematic illustration of endocytosis-related uptake pathways for NPs and all the mutants utilized in this study. (B–C) Percent mortality of N2 and endocytosis mutants 24 h post exposure to (B) $AgNO_3$ ($0.05 \text{ mg-Ag L}^{-1}$) and (C) citrate-coated AgNP (CIT-AgNPs) (1 mg-Ag L^{-1}). *indicates that the strain was significantly different from N2 (Kruskal-Wallis). For $AgNO_3$ exposure, $N = 18$ for each strain, with data pooled from 3 separate experiments. For CIT-AgNP exposure, $N = 12$ for each strain, with data pooled from 2 separate experiments. Boxplots show the 10%, 25%, median, 75% and 90% quantiles for mortality. D) Total silver content ($\pm$ standard error of the mean) in all strains upon exposure to $AgNO_3$ and CIT-AgNPs, as measured by ICP-MS. For each strain, $N = 5$ for $AgNO_3$ and $N = 6$ for CIT-AgNPs, and data were pooled from 2 separate batches of nematode samples. E) Correlation between mean mortality 24 h after the silver treatments and total silver concentration in nematodes based on nematode dry weight.

Figure 3.

Percent mortality of N2 and lysosomal mutants 24 hours post exposure to (A) AgNO₃ (0.0 – 0.12 mg-Ag L⁻¹) and (B) CIT-AgNPs (0.0 – 1.0 mg-Ag L⁻¹). Data represent 4–6 replicate experiments, with each experiment containing 6 replicate wells. No statistically significant strain differences in response to AgNO₃ or CIT-AgNPs were detected ($p > 0.05$ for significance of interaction term in 2-factor ANOVA).

Table 1

Mutant analysis (endocytosis deficient strains) for all types of AgNP exposure. + indicates baseline sensitivity; – indicates increased resistance, based on 24 h mortality and as compared to wild-type. Increased sensitivity was not observed. The AgNPs are denoted by the type of coating used during synthesis (polyvinylpyrrolidone, citrate, gum arabic) and the average particle diameter (shown as subscript in nanometers) for PVP- and GA-AgNPs.

NP	N2	<i>rme-1</i>	<i>rme-6</i>	<i>rme-8</i>
AgNO ₃	+	+	+	+
PVP ₈ -Ag NPs	+	+	+	+
PVP ₃₈ -Ag NPs	+	+	+	+
CIT-Ag NPs	+	+	–	+
GA ₅ -Ag NPs	+	+	+	+
GA ₂₂ -Ag NPs	+	+	+	+