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Hypoxia inducible factor-1 (HIF-1)–flavin containing monooxygenase-2 (FMO-2) signaling acts in silver nanoparticles and silver ion toxicity in the nematode, *Caenorhabditis elegans*

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ABSTRACT

In the present study, nanotoxicity mechanism associated with silver nanoparticles (AgNPs) exposure was investigated on the nematode, *Caenorhabditis elegans* focusing on the hypoxia response pathway. In order to test whether AgNPs-induced hypoxia inducible factor-1 (HIF-1) activation was due to hypoxia or to oxidative stress, depletion of dissolved oxygen (DO) in the test media and a rescue effect using an antioxidant were investigated, respectively. The results suggested that oxidative stress was involved in activation of the HIF-1 pathway. We then investigated the toxicological implications of HIF-1 activation by examining the HIF-1 mediated transcriptional response. Of the genes tested, increased expression of the flavin containing monooxygenase-2 (FMO-2) gene was found to be the most significant as induced by AgNPs exposure. We found that AgNPs exposure induced FMO-2 activation in a HIF-1 and p38 MAPK PMK-1 dependent manner, and oxidative stress was involved in it. We conducted all experiments to include comparison of AgNPs and $AgNO_3$ in order to evaluate whether any observed toxicity was due to dissolution or particle specific. The AgNPs and $AgNO_3$ did not produce any qualitative differences in terms of exerting toxicity in the pathways observed in this study, however, considering equal amount of silver mass, in every endpoint tested the AgNPs were found to be more toxic than $AgNO_3$. These results suggest that Ag nanotoxicity is dependent not only on dissolution of Ag ion but also on particle specific effects and HIF-1–FMO-2 pathway seems to be involved in it.

Introduction

Silver has been used for centuries in health care delivery for its antimicrobial and anti-inflammatory properties (Chaloupka et al., 2010; Lansdown, 2010). The advent of engineered AgNPs has provided novel opportunities for the use of silver in both consumer and biomedical applications, offering a larger surface area-to-volume ratio and resulting increased reactivity (Chaloupka et al., 2010; Chen and Schluesener, 2008; Wijnhoven et al., 2009). Despite recent research efforts to understand the toxicity and safety aspects of AgNPs (Ahamed et al., 2010; Wiesner et al., 2009), serious gaps in knowledge relating to Ag nanotoxicity remain, particularly regarding its mechanisms. Reactive oxygen species (ROS) generation and oxidative stress are the most developed paradigms available for explaining the toxic effects of AgNPs. Recent studies on oxidative stress associated with AgNPs exposures have focused on oxidative stress phenomena by measuring the formation of ROS, antioxidant enzyme activation, *etc.* (Foldbjerg et al., 2011; Kim et al., 2009; Piao et al., 2011), with only a few studies having ventured into the signaling pathways involved in the nanotoxic mechanism (AshaRani et al., 2009; Kang et al., 2012). However, deeper study into the mechanisms of toxicity is particularly needed for those NPs applied to biomedical purposes, such as AgNPs, as a detailed understanding of the interactions of NPs with biomolecules is crucial for the development of their use in biomedical applications.

The majority of nanotoxicity studies have been conducted using *in vitro* test systems (Arora et al., 2012; Clift et al., 2011; Lewinski et al., 2008). However, one of the fundamental approaches for determining NP safety is to relate their toxicity mechanisms, determined at the cellular level, to the whole organism. Of various *in vivo* models, the nematode *Caenorhabditis elegans*, is particularly useful for the investigation of nanotoxicity mechanisms. Because of its high degree of molecular conservation and outstanding molecular, genetic, and genomic tools available for their use with much simplicity than commonly used rodent model, *C. elegans* results can be successfully extrapolated to provide mechanistic insights into human health effects.

Previously, we reported oxidative stress as a mechanism of action associated with AgNPs exposure in *C. elegans*, including the involvement of hypoxia inducible factor-1 (HIF-1) *via* the p38 mitogen activated protein kinase (MAPK) PMK-1 pathway (Lim et al., 2012). Transcription

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factor HIF-1, a central regulator of physiological and pathological adaptation to hypoxia (Bruick, 2003; Maxwell, 2005; Semenza, 2003), is evolutionally conserved in *C. elegans* (Epstein et al., 2001; Jiang et al., 2001). Although the hypoxia pathway has been rarely studied from a toxicological context, because hypoxia is closely related to various pathological conditions, this pathway is highly suspected to be involved in toxicity response.

In this study, in order to gain understanding of the underlying mechanisms of AgNPs-induced oxidative stress, *C. elegans* HIF-1 pathway was investigated. Previously observed AgNPs-induced HIF-1 activation (Lim et al., 2012) led us to hypothesize that increased HIF-1 activity may be due to AgNPs-induced hypoxia or hypoxic response *via* ROS. To test whether or not AgNPs exposure directly created hypoxic conditions, we monitored the dissolved oxygen (DO) levels in the test media. Subsequently, to test whether or not the HIF-1 activity was due to oxidative stress, we conducted a pharmacological rescue assay on HIF-1 and other related genes in this pathway. We then investigated the physiological consequences of HIF-1 activation by examining the response of the loss-of-the function mutants of the genes involved in the regulation and function of HIF-1 pathway. We finally investigated the toxicological significance of HIF-1 activation by examining HIF-1 mediated transcriptional response, with a special focus on flavin containing monooxygenase (FMO-2) activation.

Several studies have suggested that the mechanism of Ag nanotoxicity largely depends on Ag ions (Meyer et al., 2010; Navarro et al., 2008; Xiu et al., 2011), whereas other studies have suggested that ion release does not fully explain the toxicity findings (Fabrega et al., 2009; Kim et al., 2009; Yin et al., 2011). In this study, we conducted all the experiments by comparing aspects of AgNPs and $AgNO_3$. Comparing the toxicity effects of NPs and ions allowed us to evaluate if the observed toxicity was mainly due to dissolution or if it was particle specific, as this determination is the most important characteristic in determining Ag nanotoxicity.

Materials and methods

C. elegans. *C. elegans* were grown in Petri dishes on nematode growth medium and fed with OP50 strain *Escherichia coli*, according to a standard protocol (Brenner, 1974). Worms were incubated at 20 °C, with young (3-d-old) adults from an age synchronized culture then used in all experiments. *Wildtype*(*N2*), *pmk-1*(*km25*), *hif-1*(*ia4*), *egl-9*(*sa307*), *vhl-1*(*ok161*), *fmo-2*(*ok2147*) and *cyp35a2*(*gk317*) mutants were provided by the *Caenorhabditis* Genetics Center (www.CGC.org, Supplementary Table 1) at the University of Minnesota (Minneapolis, MN, USA).

Preparation of AgNPs and exposure to C. elegans. AgNPs were prepared as described previously (Lim et al., 2012; Roh et al., 2009). Briefly, the dispersion of AgNPs was controlled by sonicating for 13 h (Branson-5210 sonicator), stirring for 7 d, and filtering through a cellulose membrane (pore size 100 nm, Advantec). From stock solutions, experimental concentrations of AgNPs and $AgNO_3$ were prepared in EPA moderately hard reconstituted water (EPA water; $NaHCO_3$ 96 mg/L, $CaSO_4\,2H_2O$ 60 mg/L, $MgSO_4$ 60 mg/L and KCl 4 mg/L). Fifty μg/L was selected as exposure level of AgNPs and $AgNO_3$, determined based on acute toxicity test (manuscript in preparation). $AgNO_3$ solution was prepared based on equal amount of silver mass.

Characterization and measurement of AgNPs and Ag ions. Particle characterization was performed in EPA water using energy-filtering transmission electron microscopy (LIBRA 120 TEM, Carl Zeiss) and photal dynamic light scattering (DLS-7000, Otsuka Electronics) for measurement of size distribution. Surface charge was investigated by zeta potential measurement using Electrophoretic Light Scattering Spectrophotometer (ELS-8000, Otsuka Electronics). The level of DO was monitored for 24 h at 20 °C in AgNPs and $AgNO_3$ solution using film-electrode DO meter (JPB-607, precision 0.1 mg/L). The concentrations of total Ag in AgNPs and $AgNO_3$ stock solution were measured using multitype inductively coupled plasma emission spectrometer (ICPE-9000, Shimadzu, Tokyo, Japan). From stock solutions, experimental concentrations 0.05 mg/L of AgNPs and $AgNO_3$ were prepared in EPA water. The Ag ion concentration in three different media (DW, EPA water, K-media) was determined in AgNPs and $AgNO_3$ solution using an Orion 4-Star pH/ISE meter (Thermo Scientific, Beverly, MA) equipped with a silver/sulfide ion-selective electrode (Orion model# 9616BNWP; Thermo Scientific) that had been calibrated against a dilution series of $AgNO_3$ solutions.

Survival and ROS formation. The survival of the *wildtype* and mutant worms were assessed after 24 h exposures to AgNPs and $AgNO_3$, as described previously (Roh et al., 2009). Briefly, survival was assessed by counting the number of live and dead individuals under a dissecting microscope while probing the worms with a platinum wire. For rescue assay, worms were pretreated for 2 h with a strong antioxidant, N-AcetylCysteine (NAC, 10 mg/L; Ates et al., 2008; Flora, 2009), before 24 h exposure with AgNPs and $AgNO_3$. ROS formation was measured as described previously (Lim et al., 2012). Briefly, *C. elegans* were exposed to AgNPs and $AgNO_3$ for 24 h, then whashed three times and transferred to 0.5 ml of S buffer, containing 30 mM 2, 7-dichlorofluoroscein diacetate (DCFHDA, Sigma). Levamisole (2 mM, Sigma-Aldrich) was applied to *C. elegans* and pictures of the live worms were then taken. The fluorescence was observed with a Leica DM IL microscope, using images obtained with a Leica DCF 420C camera.

Quantitative real time-polymerase chain reaction. For quantitative real time-polymerase chain reaction (qRT-PCR) analysis, *wildtype*(*N2*), *pmk-1*(*km25*) and *hif-1*(*ia4*) mutants were exposed to AgNPs and $AgNO_3$ for 24 h, and gene expression was analyzed using IQTM SYBR Green SuperMix (Bio-Rad). Quantitative RT-PCR was carried out on selected genes (*hif-1*, *egl-9*, *fmo-2* and *cyp35a2*) using a Chromo4 Real-Time PCR detection system (Bio-Rad). The primers were constructed based on sequences retrieved from the *C. elegans* database (www.wormbase.org) and described in Supplementary Table 2. qRT-PCR conditions were optimized and efficiency and sensitivity tests were performed for each gene prior to the main experiment. Three biological replicates were conducted for each qRT-PCR analysis.

Western blot. The HIF-1 protein expression was analyzed on the *wildtype* (*N2*), *egl-9*(*sa307*), *vhl-1*(*ok161*) mutant exposed AgNPs and $AgNO_3$ for 24 h. Western blot analysis was performed as described previously (Eom and Choi, 2009), using an enhanced chemiluminescence Western blotting detection kit (Amersham). Anti-HIF-1a was purchased from Santa Cruz Biotechnology. Anti-mouse secondary antibodies were purchased from Assay Designs.

Statistical analysis. Data are presented in arbitrary units compared to the control, with statistical differences between the *wildtype* and mutants relating to gene expression and to survival determined by an analysis of variance test, with a Tukey post-hoc test. All statistical tests were performed using Statistical Package for Social Sciences 12.0 (SPSS).

Results and discussion

Physicochemical characterization of AgNPs

In our previous studies, we used K-media as a *C. elegans* test media. However, the presence of high chloride concentration in K-media facilitates the precipitation of Ag ions by the formation of

complexes with Cl^-, which in turn reduces the toxic effects due to the Ag ions (Lim et al., 2012; Meyer et al., 2010). K-media was, therefore, deemed unsuitable for testing unstable NPs, such as AgNPs, which are prone to dissolve and release toxic ions. Alternatively, we used EPA water as test media for this study, and prior to performing the toxicity assay, physicochemical characterization of the AgNPs was conducted in EPA water to include TEM, DLS and zeta potential (Supplementary Fig. 1).

AgNPs dispersed in EPA water and measured by DLS were found to have a hydrodynamic diameter (HDD) of approximately 100–150 nm. HDD is the effective diameter of particles in liquid phase and is dependent on the salts in the liquid medium and the degree of aggregation. When particles show collective behavior (*i.e.*, group movement) in liquid media, primary particles aggregate to form secondary particles and thus, the HDD increases. The zeta potential of the AgNPs in water phase was ca. −40 mV (data not shown), but this value in EPA water was found to reduce to −25 mV. It was noted that the repulsive force induced by surface charge decreased and the distance between neighboring particles closed, leading to the formation of secondary particles. The AgNPs observed in the TEM images had a diameter of 20 nm, and the secondary AgNPs were loosely aggregated.

AgNPs and hypoxia

We previously investigated HIF-1 activation as induced by AgNPs with results suggesting that they induce hypoxic response in *C. elegans*. Hypoxia induced by chemicals has mostly been studied in order to establish chemicals for use in creating hypoxic conditions. Recently, Jiang et al. (2011) reported that sodium sulfite induced hypoxic conditions for the purpose of the development of a new chemical model for studying hypoxic injury. Therefore, in this study we investigated whether or not Ag creates a hypoxic environment in aqueous solution by measuring the DO level in the test media (Fig. 1). DO level was monitored for 24 h in media containing *C. elegans* after treatment with AgNPs and $AgNO_3$, the results for which ranged from 8.4 to 7.3 mg/L in EPA water, and from 8.2 to 7.1 mg/L in AgNPs and $AgNO_3$ solutions. The results indicated that DO level remained unchanged over a period of time in AgNPs and $AgNO_3$ containing media. These results suggested that AgNPs and $AgNO_3$ may not provoke oxygen depletion (hypoxia) in aqueous media. Thus, we then hypothesized a HIF-1 response *via* oxidative stress as a result to exposure to AgNPs and $AgNO_3$.

Oxidative stress and HIF-1

Oxidative stress is the most well-known NP induced toxicity mechanism, including toxicity from AgNPs (Mei et al., 2012; Miura and Shinohara, 2009; Wijnhoven et al., 2009), and we previously observed that oxidative stress was involved in AgNPs toxicity (Lim et al., 2012; Roh et al., 2009). The connection between HIF-1 and oxidative stress remains unclear, but a possible relationship between ROS and HIF-1 activation in stress response has been reported in cultured cells (Guzy and Schumacker, 2006; Pouysségur and Mechta-Grigoriou, 2006) and more recently in *C. elegans* (Hwang and Lee, 2011; Lee et al., 2010). It was reported that inhibition of mitochondrial respiration led to elevated ROS and increased HIF-1 activity under normal oxygen conditions (Lapointe and Hekimi, 2008; Lee et al., 2010; Sugimoto et al., 2008; Wang et al., 2010).

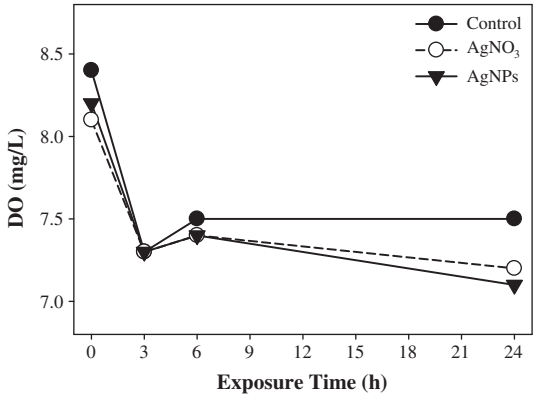

Fig. 1. Dissolved oxygen (DO) measurement. DO level was measured in EPA water treated with 0.05 mg/L of AgNPs and $AgNO_3$ for 24 h.

We previously observed that AgNPs induced ROS formation in *wildtype* nematode but not in the *hif-1* (*ia4*) mutant (Lim et al., 2012). That finding was confirmed in this study, conducted using different test media such as EPA water (Supplementary Fig. 2). We then tested the involvement of oxidative stress in AgNPs- and $AgNO_3$-induced HIF-1 activation using a pharmaceutical rescue assay (Fig. 2). We measured *hif-1* mRNA expression, both with and without treatment of NAC, in AgNPs and $AgNO_3$ exposed nematodes (Fig. 2A). Paraquat (PQ, 4 mM) was used as a positive control for oxidative stress, and as expected, NAC annulled any increased expression of the *hif-1* gene due to PQ exposure. A similar trend was observed in the AgNPs and $AgNO_3$ treated nematodes, but the increase due to AgNPs exposure was more significant than that due to $AgNO_3$, a finding suggesting that increased expression of the *hif-1* gene was due to oxidative stress. We then investigated the regulation and function of HIF-1 activation by AgNPs and $AgNO_3$, and the involvement of oxidative stress by measuring the mortality in the loss-of-function mutants of *hif-1* and its regulator genes, *egl-9* and *vhl-1*, which were exposed to AgNPs and $AgNO_3$, both with and without treatment of NAC (Fig. 2B). The EGL-9 enzyme hydroxylates a proline residue in HIF-1, the hydroxylated HIF-1 binds the VHL-1 protein, and it is then targeted for proteasomal degradation. Under hypoxic conditions, the hydroxylation reaction is inefficient and the HIF-1 is stable (Powell-Coffman, 2010). Compared to the *wildtype*, *hif-1*(*ia4*) mutant was more sensitive than *wildtype* when exposed to AgNPs but not when exposed to $AgNO_3$, a result suggesting that AgNPs-induced *hif-1* gene expression may be related to worms` survival. The mutants with functional loss of *vhl-1*(*ok161*) showed a similar level of response to AgNPs and $AgNO_3$ compared to *wildtype*, whereas the *egl-9* deficient mutants (*sa307*) were more sensitive than *wildtype* to AgNPs and to $AgNO_3$ exposure, almost to the same degree as the *hif-1*(*ia4*) mutant. A pharmacological rescue assay was also conducted. The mortality results for the nematodes exposed to AgNPs and $AgNO_3$ were reduced by treatment with NAC in all tested strains, a result strongly suggesting the involvement of oxidative stress in the regulation and function of HIF-1 response (Fig. 2B). It has been reported that *egl-9* deficient mutant is more resistant to heat, hydrogen cyanide, hydrogen sulfide and certain pathogens (Powell-Coffman, 2010). However, our results pointed in the opposite direction. In order to understand this conflict, we therefore investigated *egl-9* gene and protein expression (Fig. 3).

HIF-1 regulation by EGL-9

EGL-9 is a regulator of HIF-1 stabilization but its gene expression is reported to be regulated by HIF-1 (Shen et al., 2005). It is known that *egl-9* mRNA is induced by HIF-1 and that this establishes a negative feedback loops which attenuates HIF-1 function (Bishop et al., 2004; Epstein et al., 2001; Powell-Coffman, 2010; Shen et al., 2005). Expression of *egl-9* gene increased significantly by exposure to both AgNPs and $AgNO_3$ (Fig. 3A), which reinforced a hypothesis on a negative feedback loop for HIF-1 activation *via* EGL-9 in AgNPs and $AgNO_3$ exposure case. Compared to the *wildtype*, increased sensitivity of *egl-9* (*sa307*) mutant to AgNPs and $AgNO_3$, as observed in the *hif-1*(*ia4*) mutant (Fig. 2B) insinuated other mechanism in HIF-1 regulation, besides pathways *via vhl-1* and *egl-9*. We therefore further investigated

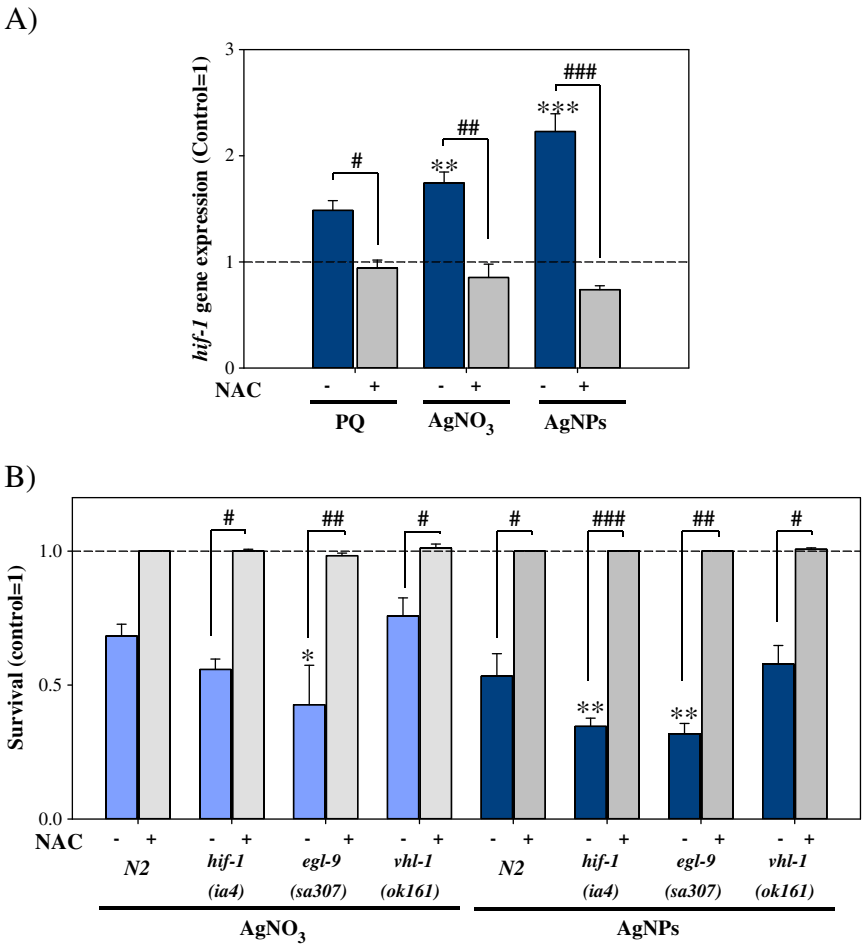

Fig. 2. The expressions of the *hif-1* gene (A) and survival rate (B) exposed to 0.05 mg/L of AgNPs and $AgNO_3$. The expression of *hif-1* gene was measured in *wildtype*(*N2*) exposed to AgNPs and $AgNO_3$ treated with or without NAC using qRT-PCR. Paraquat was used as the positive control (A). Survival analysis was conducted on *wildtype*(*N2*), *hif-1*(*ia4*), *egl-9*(*sa307*) and *vhl-1*(*ok161*) (B). The results are expressed as the mean value compared to control (control = 1, n = 3; mean ± standard error of the mean; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). The statistical difference on NAC treatment was also analyzed ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$).

HIF-1 protein expression in *wildtype* (*N2*), *egl-9*(*sa307*) and *vhl-1*(*ok161*) mutants, to understand the role of EGL-9 in AgNPs and $AgNO_3$ induced HIF-1 activation (Fig. 3B).

We found increased expression of HIF-1 at both gene (Fig. 2A) and protein levels (Fig. 3B) in *wildtype* exposed to both AgNPs and $AgNO_3$. The result suggests the role of HIF-1 in the defense of AgNPs and $AgNO_3$ exposure was due to *de novo* synthesis of HIF-1. Moreover, dramatic increase of HIF-1 by AgNPs and only limited increase by $AgNO_3$ suggest importance of HIF-1 in AgNPs toxicity over $AgNO_3$. It has been shown that HIF-1 protein level increased in *egl-9* and *vhl-1* deficient mutants (Epstein et al., 2001; Shen et al., 2005), which was also confirmed in our study, the basal level of HIF-1 is higher in *egl-9*(*sa307*) and *vhl-1*(*ok161*) mutant than in *wildtype* (*N2*) (Fig. 3B). Exposure of AgNPs and $AgNO_3$ induced expression of HIF-1 protein in *vhl-1*(*ok161*) mutant however significantly inhibit the expression in *egl-9* (*sa307*) mutants, which clearly suggest distinct pathway may exist in HIF-1 stabilization *via egl-9* and *vhl-1* at least in response to AgNPs and $AgNO_3$. This result also gives us a hint to understand why *egl-9* (*sa307*) mutant was as sensitive as *hif-1*(*ia4*) mutants to AgNPs and $AgNO_3$ exposure and *vhl-1*(*ok161*) mutant was more resistant (Fig. 2B).

This antagonistic action between EGL-9 and VHL-1 on HIF-1 regulation is also reported by Ackerman and Gems (2012) in their recent study on hypoxia signaling on iron homeostasis in *C. elegans*. In that study, loss of *vhl-1* decreases expression of GFP of *ferritin-1*, a gene involved in iron homeostasis, which was expected, given that HIF-1 represses *ftn-1* expression and that loss of *vhl-1* increases HIF-1 levels. Though they expected that loss of *egl-9*, like that of *vhl-1*, would reduce *Pftn-1::gfp* expression, they found that deletion of *egl-9* caused an increase in *Pftn-1::gfp* expression. They suggest EGL-9 inhibits *ftn-1* expression by stimulating HIF-1 activity *via* an unidentified pathway.

HIF-1 mediated transcriptional response, fmo-2, xenobiotic metabolisms enzyme

HIF-1-mediated transcriptional response has been shown to be crucial for many physiological processes including stress response (Kaelin and Ratcliffe, 2008; Leiser and Kaeberlein, 2010; Pocock and Hobert, 2008; Semenza, 2003). In the next step, we therefore investigated HIF-1 mediated transcriptional response, in order to gain insight into the physiological/toxicological consequences of HIF-1 activation by AgNPs. We investigated the expression of genes known to be regulated by *hif-1* such as the flavin monooxygenase gene-2 (*fmo-2*), nuclear hormone receptor (*nhr-57*) and *F22B5.4*, which were identified from a microarray study by Shen et al. (2005). We found increased expression of *fmo-2* gene resulting from AgNPs exposure was the most significant of those examined (about 5 times greater than the control, Supplementary Fig. 3) FMO-2 encodes the flavin-containing NADPH-dependent monooxygenases, which is involved in the oxidation of many drugs and xenobiotic and catalyzes the oxidation of glutathione (GSH) to glutathione disulfide (GSSH) (Henderson et al., 2004; Ziegler, 2002). As *fmo-2* has particular

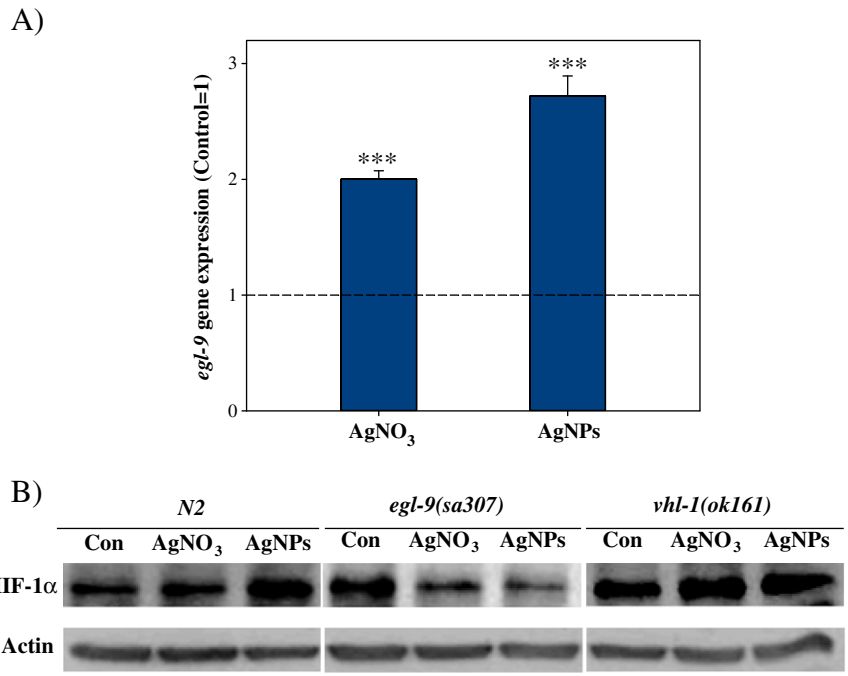

Fig. 3. The expression of the *egl-9* gene (A) and HIF-1 protein expression (B) exposed to 0.05 mg/L of AgNPs and $AgNO_3$. The expression of *egl-9* gene in *wildtype*(*N2*) using qRT-PCR and the results are expressed as the mean value compared to control (control = 1, n = 3; mean ± standard error of the mean; ***$p < 0.001$) (A). Protein expression was measured *wildtype*(*N2*), *egl-9*(*sa307*) and *vhl-1*(*ok161*) using anti-HIF-1α by Western blot (B).

importance in toxicology, we paid special attention to the up-regulation of *fmo-2* by AgNPs and $AgNO_3$ exposure.

To test whether or not the expression of *fmo-2* is regulated by HIF-1, *fmo-2* gene expression was investigated in *hif-1*(*ia4*) and *pmk-1*(*km25*) mutants (Fig. 4A), as PMK-1 was previously found to be an upstream regulator of HIF-1 (Lim et al., 2012). The result suggests that HIF-1 and PMK-1 are needed for AgNPs- and $AgNO_3$-induced *fmo-2* gene expression (Fig. 4A). However, Lee et al. (2010) found that *fmo-2* was expressed independently of *hif-1* in an experiment using two mutants defective in mitochondrial respiration: *clk-1*(*qm30*), which encodes a mitochondrial hydroxylase required for ubiquinone production, and *isp-1*(*qm150*), which encodes an iron-sulfur protein complex III. They found increased expression of *nhr-57* gene by PQ exposure (Lee et al., 2010), which was not observed in our study. These results indicated that Ag exposure and respiration inhibition may activate distinct patterns of *hif-1*-dependent gene expression. The pharmaceuticals rescue assay using NAC showed that the toxicity of *fmo-2* was significantly rescued, a finding which suggests that AgNPs exposure induced the PMK-1–HIF-1–FMO-2 pathway which is dependent on oxidative stress (Fig. 4B).

In order to further understand the toxicological significance of increased expression of the *fmo-2* gene, we additionally investigated the involvement of cytochrome P450 (CYP450) in relation to AgNPs and $AgNO_3$ exposure in order to compare its response with that of FMO, as CYP450 is a major contributor to oxidative xenobiotic metabolism and its role in xenobiotic metabolism is better understood (Fig. 5). Indeed, while much has been done to characterize CYP450, limited work has focused on FMOs (Krueger et al., 2002, 2005; Ziegler, 2002), and much less work has focused on *C. elegans*. At least 86 genes in the *C. elegans* genome encode CYP450 proteins (Lindblom and Dodd, 2006; Menzel et al., 2001), whereas 5 FMOs (fmo-1 to fimo-5) are identified in the *C. elegans* genome (Petalcorin et al., 2005). Gene expression patterns for five *C. elegans* FMOs reported by Petalcorin et al. (2005) indicate that the genes are expressed intestinally (*fmo-1*, *-2* and *-5*) or hypodermally (*fmo-3*, and *-4*), and that both expression patterns may reflect detoxifying roles for nematode FMOs as these tissues represent initial exposure points for xenobiotics in food and/or from their external environment. In the same study, they also reported that the nematode's FMOs were inducible by exposure to benzydamine, a xenobiotic FMO substrate.

Of the CYPs, we tested the *cyp35a2* gene as the *C. elegans* CYP35 genes are responsive to a variety of xenobiotic stressors (Menzel et al., 2001, 2005; Reichert and Menzel, 2005). And in our previous studies using metals (Cd and As; Roh et al., 2006), organic compounds (DEHP, OP; Roh and Choi, 2011; Roh et al., 2007), and NPs (CeO_2 and TiO_2; Roh et al., 2010), increased expression of this gene were observed. AgNPs and $AgNO_3$ exposure also induced expression of the *cyp35a2* gene, only with less intensity than observed for *fmo-2* (Fig. 5A). Surprisingly, the loss-of-function *cyp35a2*(*gk317*) mutant did not show any difference in terms of mortality resulting from AgNPs or $AgNO_3$ exposure as compared to the *wild-type*, a finding which was significantly different than the response of the *fmo-2*(*ok2147*) mutant, whose sensitivity increased dramatically as compared to the *wildtype* (Fig. 5B). These results showed that the transcriptional activation of *fmo-2* led to a functional consequence, whereas that of *cyp35a2* did not, a finding suggesting the greater importance of FMO over CYP450 in Ag metabolism. Importance of FMO-2 in AgNPs toxicity was already pointed out in a nanotoxicity study using mouse brain (Rahman et al., 2009). They reported upregulation of *fmo-2* gene by AgNPs explaining as an involvement in metabolic protection from AgNPs-induced oxidative stress and further suggest this may disturb GSSG/GSH balance. It is also interesting to note that *fmo-2* gene was included in most differentially expressed genes (DEGs) annotated to one of the top ten pathways connecting the most DEGs by complex network analysis (Roca et al., 2012), which was conducted on our previously published microarray dataset on AgNPs-exposed *C. elegans* (GSE14932 in Roh et al., 2009).

Dissolution of silver ion

In this study we conducted all the experiments as comparisons between AgNPs and $AgNO_3$. In comparing the effects of AgNPs and

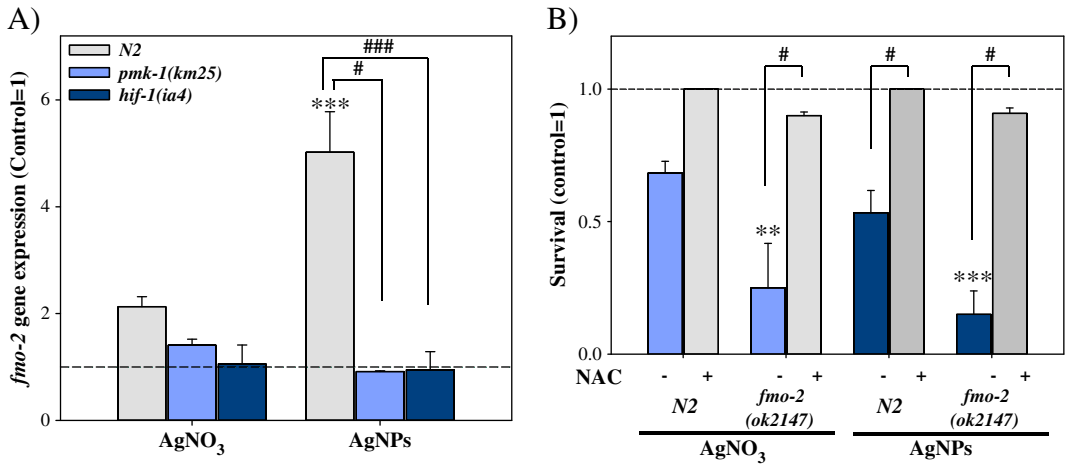

Fig. 4. The expression of the *fmo-2* gene (A) and survival rate (B) exposed to 0.05 mg/L of AgNPs and $AgNO_3$. The expression of *fmo-2* gene was measured in *wildtype*(*N2*), *pmk-1*(*km25*) and *hif-1*(*ia4*) using qRT-PCR and the results are expressed as the mean value compared to control (control = 1, n = 3; mean ± standard error of the mean; $^{***}p < 0.001$). The statistical difference between *wildtype*(*N2*) and mutants was analyzed ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$) (A). Survival analysis was conducted on *wildtype*(*N2*) and *fmo-2*(*ok2147*) mutants exposed to AgNPs and $AgNO_3$ and the results were expressed as the mean value compared to control (control = 1, n = 3; mean standard error of the mean; $^{**}p < 0.01$, $^{***}p < 0.001$). The statistical difference on NAC treatment was also analyzed ($^{\#}p < 0.05$) (B).

$AgNO_3$, the overall results consistently showed that the toxic response was greater with the AgNPs than with the $AgNO_3$ (Figs. 2–5; Statistic significance of each tested endpoint are shown in Supplementary Table 3). As toxic ion release was the most important characteristic in determining the Ag nanotoxicity, even though it was naturally expected that the ion release rate would be greater for $AgNO_3$ than AgNPs, Ag ion concentrations were measured in AgNPs and $AgNO_3$ solution after spiking 1 mg/L of Ag at the beginning of the exposure (Fig. 6). Chloride has been known to decrease Ag ion toxicity by precipitation of Ag ions to AgCl (Hogstrand and Wood, 1998), therefore, to test effect of chloride concentrations on Ag ion release, measurement was performed in three different media with different level of chloride, which were distilled water (DW, Fig. 6A), EPA water (Fig. 6B) and K-media. As expected, the concentration of Ag ion was higher in DW than in EPA water, and $AgNO_3$ released more ions than AgNPs did, whereas, Ag ion was not detected at all in both AgNPs and $AgNO_3$ solution prepared in K-media. Lee et al. (2012) investigated the release kinetics of Ag ions from AgNPs and found the concentration of Ag ion increased exponentially within the first 6 h. Our result showed that AgNPs solution (both in DW and EPA water) contains high level of Ag ions, even at the beginning of the experiment. This was also observed in AgNPs stock solutions (10 mg/L), in which Ag ions level was nearly 6 mg/L (Supplementary Fig. 4). In our study, AgNPs solution was prepared by dispersion in water using 13 h sonication followed by 7 days stirring. This long preparation time is enough to make Ag ion fully dissolved from AgNPs, which may be an explanation of high Ag ion concentration at the beginning of the experiment. Even though our AgNPs solution contains high level of Ag ion, our dissolution experiment clearly showed difference of Ag ion concentration between AgNPs and $AgNO_3$ solution (Fig. 6). This result suggests that our toxicity results whereby the response was constitutively greater when the nematodes were exposed to AgNPs than to $AgNO_3$ (Figs. 2–5; Supplementary Table 3) were due to particle rather than dissolved ions.

AgNPs and Ag ion toxicities have been compared and analyzed in numerous publications, but the mechanism of AgNPs toxicity remains unclear, mostly because the toxicity studies were conducted with apical endpoints, *i.e.* cytotoxicity for *in vitro* studies and mortality or growth for *in vivo* studies (Choi et al., 2011; Ellegaard-Jensen et al., 2012; Lee et al., 2012). Few studies have been performed with the analysis of deeper mechanistic values as the goal. Although much

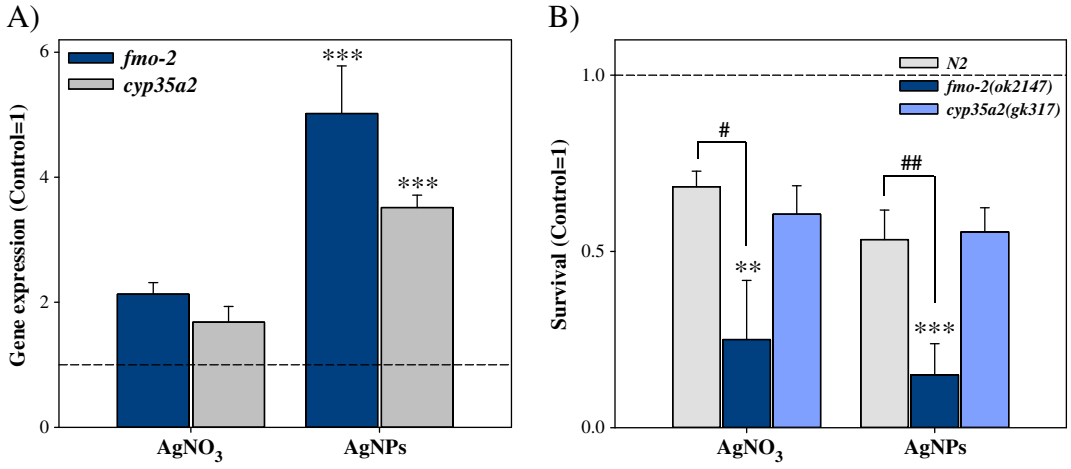

Fig. 5. The expressions of the *fmo-2* and *cyp35a2* genes (A) and survival rate (B) exposed to 0.05 mg/L of AgNPs and $AgNO_3$. The expression of *fmo-2* and *cyp35a2* genes was measured in *wildtype*(*N2*) exposed to AgNPs and $AgNO_3$ using qRT-PCR (A). Survival analysis was conducted on *wildtype*(*N2*), *fmo-2*(*ok2147*) and *cyp35a2*(*gk317*) exposed to AgNPs and $AgNO_3$. The results are expressed as the mean value compared to the control (control = 1, n = 3; mean ± standard error of the mean; $^{**}p < 0.01$, $^{***}p < 0.001$). The statistical difference between *wildtype*(*N2*) and mutants was also analyzed ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$) (B).

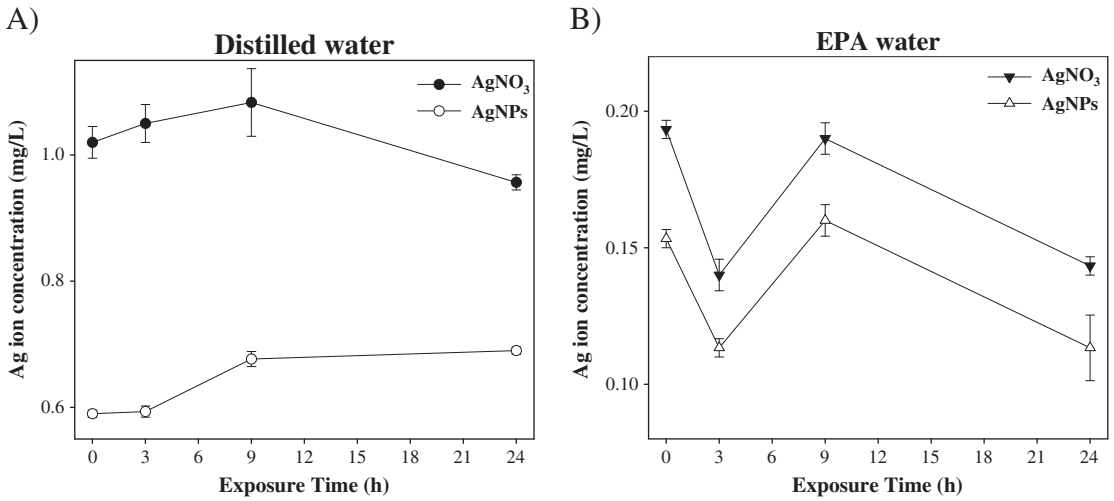

Fig. 6. Concentration of Ag ion measured for 24 h in distilled water (A) and EPA water (B) after spiking 1 mg/L of AgNPs and $AgNO_3$. Ag ion concentration was not detected in K-media due to high ionic strength.

more on this topic needs investigation, our results provided insight on the contribution of Ag ion and AgNPs specific effects on oxidative stress dependent HIF-1–FMO-2 signaling pathway in *C. elegans*.

Conclusion

Overall results suggest that Ag nanotoxicity is dependent on Ag ion release, however, this alone does not explain all aspects of the Ag nanotoxicity. Even with the limitations of our study design, our results provided useful information for understanding the PMK-1–HIF-1–FMO-2 pathway as a mechanism of AgNPs-induced toxicity in *C. elegans*. Our study produced five important conclusions: 1) The prolyl hydroxylase EGL-9, which represses HIF-1 *via* VHL-1, can also act antagonistically to VHL-1 in response to AgNPs; 2) FMO-2 is significantly upregulated by AgNPs in PMK-1 and HIF-1 dependent manner, suggesting that the PMK-1–HIF-1–FMO-2 pathway is involved in Ag nanotoxicity; 3) AgNPs and $AgNO_3$ induced *pmk-1*, *hif-1* and *fmo-2* gene expression and increased mortality in their loss-of-function mutants, a finding which suggests transcriptional activation of these genes leads to toxicological consequences; 4) Toxicity of AgNPs and $AgNO_3$ (*i.e.* increased expression of *pmk-1*, *hif-1* and *fmo-2*, and increased sensitivity of their mutants) was rescued by treatment with NAC, a finding suggesting that oxidative stress was involved in their toxic effects; 5) Neither AgNPs nor $AgNO_3$ revealed any qualitative difference in terms of exerting toxicity in the pathways observed in this study, however, in every endpoint we found, the AgNPs were more toxic than an equal amount of silver mass contained in $AgNO_3$.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.03.028.

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