SETAC PRESS

Environmental Toxicology and Chemistry
© 2011 SETAC
Printed in the USA
DOI: 10.1002/etc.1706

Environmental Toxicology

OXIDATIVE STRESS-RELATED PMK-1 P38 MAPK ACTIVATION AS A MECHANISM FOR TOXICITY OF SILVER NANOPARTICLES TO REPRODUCTION IN THE NEMATODE *CAENORHABDITIS ELEGANS*

DONGYOUNG LIM,† JI-YEON ROH,† HYUN-JEONG EOM,† JEONG-YUN CHOI,‡ JINWON HYUN,§ and JINHEE CHOI*†
†School of Environmental Engineering, Graduate School of Energy and Environment Systems Engineering, University of Seoul, Seoul, Korea
‡Division of Pharmacology, Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, Korea
§School of Medicine and Applied Radiological Research Institute, Jeju National University, Jeju, Korea

(*Submitted* 10 *June* 2011; *Returned for Revision* 25 *July* 2011; *Accepted* 9 *October* 2011)

Abstract—In the present study, a toxic mechanism of silver nanoparticles (AgNPs) was investigated in the nematode, *Caenorhabditis elegans*, focusing on the involvement of oxidative stress in reproduction toxicity. Initially, AgNPs were tested as potential oxidative stress inducers, and increased formation of reactive oxygen species (ROS) was observed in AgNP-exposed *C. elegans*. Subsequently, the potential upstream signaling pathway activated in response to AgNP exposure was investigated, paying special attention to the *C. elegans* PMK-1 p38 mitogen-activated protein kinase (MAPK). Increased PMK-1 p38 MAPK gene and protein expressions were observed in *C. elegans* exposed to AgNPs. Expression of the p38-dependent transcription factor genes and glutathione *S*-transferase (GST) enzyme activity was also investigated in *wildtype (N2)* and *pmk-1* mutant *(km25) C. elegans* exposed to AgNPs. The results indicated that AgNP exposure led to increased ROS formation, increased expression of PMK-1 p38 MAPK and hypoxia-inducible factor (HIF-1), GST enzyme activity, and decreased reproductive potential in *wildtype (N2) C. elegans*; whereas in the AgNP-exposed *pmk-1 (km25)* mutant, ROS formation and HIF-1 and GST activation were not observed, and decreased reproductive potential was rescued. These results suggest that oxidative stress is an important mechanism of AgNP-induced reproduction toxicity in *C. elegans,* and that PMK-1 p38 MAPK plays an important role in it. The results also suggest that GST and HIF-1 activation by AgNP exposure are PMK-1 p38 MAPK–dependent, and that both play an important role in the PMK-1 p38 MAPK-mediated defense pathway to AgNP exposure in *C. elegans*. Environ. Toxicol. Chem. © 2011 SETAC

Keywords—Silver nanoparticles *Caenorhabditis elegans* Reproduction Oxidative stress Mitogen-activated protein kinase

INTRODUCTION

Due to the wide application of nanoparticles (NPs) in various fields, research on their toxicity has grown exponentially over the past few years [1]. Nevertheless, knowledge in this area is still seriously deficient, especially regarding the mechanisms of NPs. Numerous previous studies have reported oxidative stress as one of the most important toxicity mechanisms related to NP exposure [2]. Silver nanoparticles (AgNPs) are used in increasing numbers of products, and given the potentially high toxicity and specific concerns associated with the use of AgNPs, particular attention to their toxicity may be warranted. Recent studies on the toxicity and risk of AgNPs suggest that, directly or indirectly, oxidative stress may be a potential toxic mechanism, because AgNP exposure can trigger oxidative stress or exacerbate pre-existing oxidant reactions, thereby inducing toxicity [3,4]. However, even though numerous recent studies have suggested that AgNPs may exert their toxicity through oxidative stress, the upstream signaling mechanism responsible for regulating the oxidative stress by AgNPs is still poorly understood, especially in non-mammalian models, such as *Caenorhabditis elegans*. The nematode *C. elegans* is an excellent model organism for researching and assessing environmental contaminants, and particularly for studying the ecotoxicological relevance of chemical-induced molecular-level responses; comprehensive knowledge of the genome of *C. elegans* and of functional genomics tools allows for a clearer insight into the operation of toxic mechanisms initiated by chemicals acting upon organisms, mechanisms that can have adverse effects at the population level.

In our previous study, AgNPs were observed to induce serious alterations to reproductive potential in *C. elegans*; significantly, increased expression of stress-related genes in the *sod-3(gk235)* mutant suggested that oxidative stress may be involved in AgNP toxicity in *C. elegans* [5]. In the present study, continuing our previous work, oxidative stress was investigated as an underlying mechanism of AgNP-induced reproduction toxicity in *C. elegans.* It is known that mitogen-activated protein kinases (MAPKs) serve as transducers of extracellular stimuli, which play key roles in diverse physiological processes, including stress response [6]. A *C. elegans* ortholog of the p38 MAPK, PMK-1 is known to play a critical role in the response against oxidative stress and innate immunity in *C. elegans* [7,8]. Although the roles of the PMK-1 p38 MAPK pathways in various stress responses of *C. elegans* have already been reported [9,10], they have rarely been approached in an ecotoxicological context.

In the present study, to test whether oxidative stress was directly involved in AgNP toxicity, reactive oxygen species (ROS) was measured in AgNP-exposed *C. elegans.* Subsequently, the potential upstream signaling pathways activated in response to AgNPs were investigated, with special attention paid to the *C. elegans* PMK-1 p38 MAPK signaling pathway. The expression of hypoxia inducible factor-1 (HIF-1) was investigated as a PMK-1 p38 MAPK-dependent transcription factor (TF) in response to AgNP exposure. Finally, the functional consequences of activating the PMK-1 p38 MAPK

All Supplemental Data may be found in the online version of this article.
* To whom correspondence may be addressed
(jinhchoi@uos.ac.kr).
Published online 29 November 2011 in Wiley Online Library
(wileyonlinelibrary.com).

pathway in response to AgNPs were investigated using glutathione *S*-transferase (GST) activity and reproductive potential, as well as ROS formation.

MATERIALS AND METHODS

Maintenance and treatment of AgNPs to C. elegans

Caenorhabditis elegans were grown in Petri dishes on nematode growth medium and fed with OP50 strain *Escherichia coli,* according to a standard protocol [11]. Worms were incubated at 20°C, with young (3-d-old) adults from an age-synchronized culture then used in all experiments. *Wildtype (N2)* and *pmk-1 (km25)* mutants were provided by the *Caenorhabditis* Genetics Center (www.CGC.org; Supplemental Data, Table S1) at the University of Minnesota (Minneapolis, MN, USA). For use as treatment for *C. elegans*, AgNPs were prepared and characterized as described previously by Roh et al. [5]. 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) [12]. The size distribution of the AgNPs was examined by energy-filtering transmission electron microscopy (LIBRA 120 TEM, Carl Zeiss) and photal dynamic light scattering (DLS-7000, Otsuka Electronics). From stock solutions, experimental concentrations (0.1, 0.5, and 1 mg/L) of AgNPs and $AgNO_3$ were prepared in k-media (0.032 M KCl and 0.051 M NaCl).

ROS formation

To detect the levels of reactive oxygen species (ROS), *wildtype (N2)*, *pmk-1 (km25), hif-1* RNA interference (RNAi), and *pmk-1 (km25);hif-1* (RNAi) *C. elegans* were exposed to 1 mg/L of AgNPs for 24 h, then transferred to 0.5 ml of S buffer, containing 30 μM 2, 7-dichlorofluoroscein diacetate (DCFH-DA, Sigma), a well-established compound used for detecting and quantifying ROS [13,14], for preincubation for 30 min at 20°C. H_2O_2 (0.01 M, 30 min) was used as a positive control. To confirm the role of ROS, worms were pretreated with a strong antioxidant, 300 μM N-acetylcysteine, for 2 h before AgNP exposure [15,16]. The fluorescence was observed with a Leica DM IL microscope, using images obtained with a Leica DCF 420C camera. Levamisole (2 mM, Sigma-Aldrich) was applied to *C. elegans;* pictures of the live worms were then taken. Image analyses were conducted on five individual biological replications.

Quantitative real time-polymerase chain reaction

For quantitative real time-polymerase chain reaction (qRT-PCR) analysis, *wildtype (N2)* and *pmk-1 (km25)* mutant were exposed to 0.1, 0.5, and 1 mg/L of AgNPs for 4 and 24 h, and then analyzed using IQTM SYBR Green SuperMix (Bio-Rad). Quantitative RT-PCR was carried out on selected genes 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; Supplemental Data, Table S2), qRT-PCR conditions optimized, and efficiency and sensitivity tests performed for each gene prior to the main experiment. Three biological replicates were conducted for each qRT-PCR analysis.

pmk-1::gfp *measurement*

Transgenic *C. elegans* were incubated for 4 and 24 h with 1 mg/L of AgNPs. Worms were mounted on 5% agar pads on a glass slide, immobilized in 2 mM levamisole (Sigma-Aldrich). The fluorescence signal emitted from *pmk-1::gfp* transgenic worms was detected using a Leica DM IL microscope. Five individual biological replications were conducted for each treatment.

Western blot

P38 MAPK protein expression were analyzed on the *wildtype (N2)* exposed to 0.1, 0.5, and 1 mg/L of AgNPs for 24 h, with those exposed to 1 mg/L of AgNPs exposed for 1, 2, 4, 12, 24, and 48 h. The HIF-1 protein expression was analyzed on the *wildtype (N2)* and *pmk-1 (km25)* mutant exposed to 0.1, 0.5, and 1 mg/L of AgNPs for 24 h. Western blot analysis was performed as described previously [17], using an enhanced chemiluminescence Western blotting detection kit (Amersham). Anti-p38, p-p38 was obtained from Assay Designs Cell Signaling and Anti-HIF-1α was purchased from Santa Cruz Biotechnology. Anti-rabbit secondary antibodies were purchased from Assay Designs. Three replicates were conducted for each treatment, along with a control. Following Western blotting, the relative densities of the protein bands were determined using an image analyzer, the Gel Documentation system (Vilber Lourmat TFX-20.M), coupled to a Kodak 1D 3.6 camera (Kodak EDAS 290).

GST enzyme activity

Glutathione *S*-transferases enzyme activity analyses were conducted spectrophotometrically (Thermo Scientific, EVO 60) through measurement of glutathione-1-chloro-2,4-dinitrobenzene (CDNB) conjugate production [18] on the *wildtype (N2)*, *pmk-1 (km25)*, *hif-1* (RNAi), and *pmk-1 (km25);hif-1* (RNAi) *C. elegans* exposed to 5 mg/L of AgNPs for 24 h.

hif-1 *RNA interference*

The *hif-1* RNA interference (RNAi) feeding was performed on *wildtype (N2)* and on *pmk-1 (km25)* mutant *C. elegans*, as previously described by Roh et al. [19]. The RNAi bacteria were induced for 48 h at room temperature for dsRNA expression. We added L3- to L4-stage animals to the plate and incubated at 20°C. After 36 to 40 h, worms were transferred to another large-scale plate seeded with the same RNAi bacteria, and then were allowed to grow to adulthood and lay eggs. When worms were fully grown, adults were removed by the age-synchronized culture method. Each egg was seeded to new, freshly prepared RNAi feeding plates. To evaluate the efficiency of the dsRNA feeding, more than 1,000 worms were assessed by semiquantitative PCR (Supplemental Data, Fig. S1).

Reproduction assay

The effects of AgNPs on the reproduction of *wildtype (N2)*, *pmk-1 (km25), hif-1* (RNAi), and *pmk-1 (km25);hif-1*(RNAi) *C. elegans* were investigated by counting the number of offspring from one early-stage young adult 72 h after exposure to 0.1 mg/L of AgNPs, as described previously by Roh et al. [5].

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 reproduction determined by an analysis of variance test, with a Dunnett's multiple comparison test. All statistical tests were performed using Statistical Package for Social Sciences 12.0 (SPSS).

RESULTS

Because the physicochemical attributes of NPs are critical parameters in determining their degree of toxicity, the characterization of AgNPs was investigated, as described previously [5], prior to the toxicity experiments. The characterization data confirmed that the sizes of particles applied in the toxicity test were mainly within the range of 20 to 30 nm, and were evenly distributed in the test medium (Supplemental Data, Fig. S2).

Oxidative stress has been reported frequently as a major toxic mechanism of AgNPs, which was also suggested in our previous study on *C. elegans* [5]. In the present study, to obtain direct evidence of this suggestion, ROS formation was monitored in *C. elegans*. Increased fluorescence intensity was observed in AgNP-exposed *wildtype (N2) C. elegans,* but this was suppressed by pretreatment with N-acetylcysteine, an important antioxidant, which confirms that the AgNP-induced increase in ROS formation was due to oxidative stress (Supplemental Data, Fig. S3).

In our previous study, it was also reported that AgNPs were more toxic than $AgNO_3$ and that the genes altered by AgNPs differed from those altered by $AgNO_3$, suggesting that different mechanisms may be involved in their toxicities [5]. Reactive oxygen species formation and reproductive potential were investigated in $AgNO_3$- and AgNP-exposed *wildtype (N2)* and various stress-response mutants of *C. elegans* (data not shown). Of the responses of the mutants, those of *pmk-1 (km25)* are presented in Supplemental Data, Figure S4. Silver nanoparticles led to increased ROS formation and decreased reproductive potential in *wildtype (N2) C. elegans*, whereas ROS formation and reproductive potential were both rescued in the *pmk-1 (km25)* mutant. However, decreased reproductive potential by $AgNO_3$ in *wildtype (N2) C. elegans* was not rescued in *pmk-1 (km25)* mutant, and ROS formation was not observed in $AgNO_3$-exposed worms.

The results in Supplemental Data, Figure S4 suggest the involvement of PMK-1 p38 MAPK in the oxidative stress induced by AgNPs in *C. elegans*; therefore, we investigated *pmk-1* gene and *pmk-1::gfp* expression in AgNP-exposed worms to test whether this is activated at the transcriptional level (Fig. 1). Expression of the *pmk-1* gene was observed to be increased by AgNP exposure, suggesting transcriptional activation (Fig. 1A). This was also confirmed by the response of *pmk-1::gfp C. elegans* on exposure to AgNPs. Fluorescent intensity in *pmk-1::gfp C. elegans* was observed to increase on exposure to AgNPs, suggesting that the *pmk-1* promoter was activated on exposure to AgNPs (Fig. 1B). In the next step, we investigated whether expression of the *pmk-1* gene led to protein expression of p38, using a Western blot analysis, on AgNP-exposed *wildtype (N2) C. elegans.* Because it is known

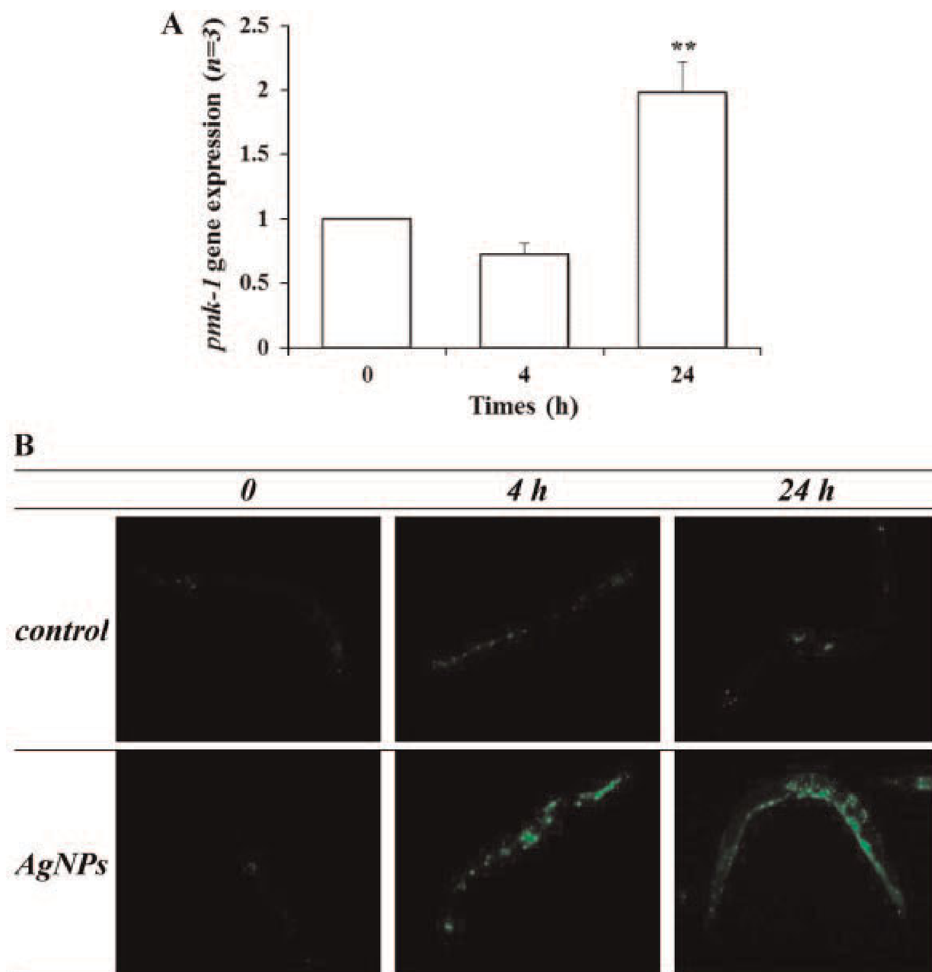

Fig. 1. The expression of the *pmk-1* gene (**A**) and *pmk-1::gfp* (**B**) on exposure to silver nanoparticles (AgNPs). The *pmk-1* gene and *pmk-1::gfp* expressions in *wildtype (N2)* exposed to 1 mg/L of AgNPs for 0, 4, and 24 h. Quantitative real time-polymerase chain reaction was performed for a gene expression analysis. The results are expressed as the mean value compared to the control (control = 1; $n = 3$; mean ± standard error of the mean using a one-way analysis of variance test [$^{**}p < 0.01$]). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

that the activity of p38 kinase is critical for functional activation, this was confirmed using the phosphorylated p38 antibody (Fig. 2). The results showed that expression of p38 and phosphorylated p38 increased at 0.5 and 1 mg/L of AgNP-exposed *C. elegans*, as early as 1 h after exposure until 48 h, suggesting a functional role of p38 in the toxicity of AgNPs.

To identify the genes potentially involved in the PMK-1 p38 MAPK signaling pathway, SKiNhead-*1 (skn-1)*, abnormal DAuer Formation-16 *(daf-16)*, *C. elegans* P-53-like protein-1 *(cep-1),* and *hif-1* were selected, as they are well-known stress-response TFs in *C. elegans* (Fig. 3). Of the TFs tested, AgNPs appeared to induce *hif-1* gene expression in *wildtype (N2)* worms. Therefore, to investigate whether this was dependent on PMK-1 p38 MAPK, *hif-1* gene expression was measured also in the *pmk-1 (km25)* mutant (Fig. 4A). After exposure to AgNPs, expression of *hif-1* was observed to be dramatically increased in the *wildtype (N2)* worm but not in the *pmk-1 (km25)* mutant, suggesting that AgNP-induced *hif-1* gene expression may be dependent on PMK-1 p38 MAPK. To elucidate whether PMK-1 p38 MAPK-dependent *hif-1* gene expression also occurs at the protein level, Western blot analyses were conducted on *wildtype (N2)* and the *pmk-1 (km25)* mutant strain (Fig. 4B). The results revealed that HIF-1 protein expression was also induced by AgNPs in *wildtype (N2) C. elegans*, but not in the *pmk-1 (km25)* mutant.

When activation of PMK-1 p38 MAPK and HIF-1 due to exposure to AgNPs had been confirmed, the responses of reproductive potential (Fig. 5A), GST activity (Fig. 5B), and ROS formation (Fig. 5C) to AgNP exposure were investigated in *C. elegans*, to address the functional consequences of the activation of these signaling proteins. These experiments were conducted using *wildtype (N2)*, *pmk-1 (km25)*, *hif-1* (RNAi), and *pmk-1 (km25);hif-1* (RNAi) *C. elegans* after exposure to AgNPs. Increased GST activity and ROS formation were observed on exposure to AgNPs in *wildtype (N2), hif-1* (RNAi), and *pmk-1 (km25);hif-1* (RNAi) worms, but not in the *pmk-1 (km25)* single mutant. Decreased reproductive potential by AgNP exposure was observed in all four strains, but *pmk-1 (km25)* was less affected than the three other strains.

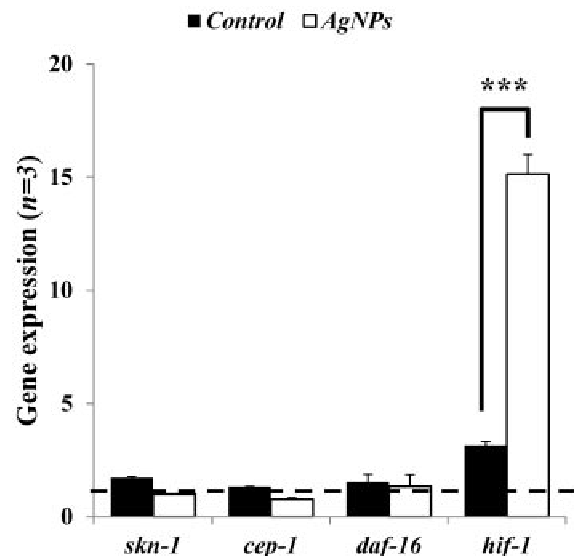

Fig. 3. The expression of transcription factor genes (*skn-1, cep-1, daf-16, and hif-1*) in *wildtype (N2)* exposed to 1 mg/L of silver nanoparticles (AgNPs) for 24 h. Quantitative real time-polymerase chain reaction was performed for a gene expression analysis ($n = 3$; mean $\pm$ standard error of the mean using a one-way analysis of variance test [$^{***}p < 0.001$]).

DISCUSSION

It is hypothesized that the toxic effects of AgNPs are due to a combination of the specific properties of AgNPs and the generation of their ions [3]. Our previous study also suggested that

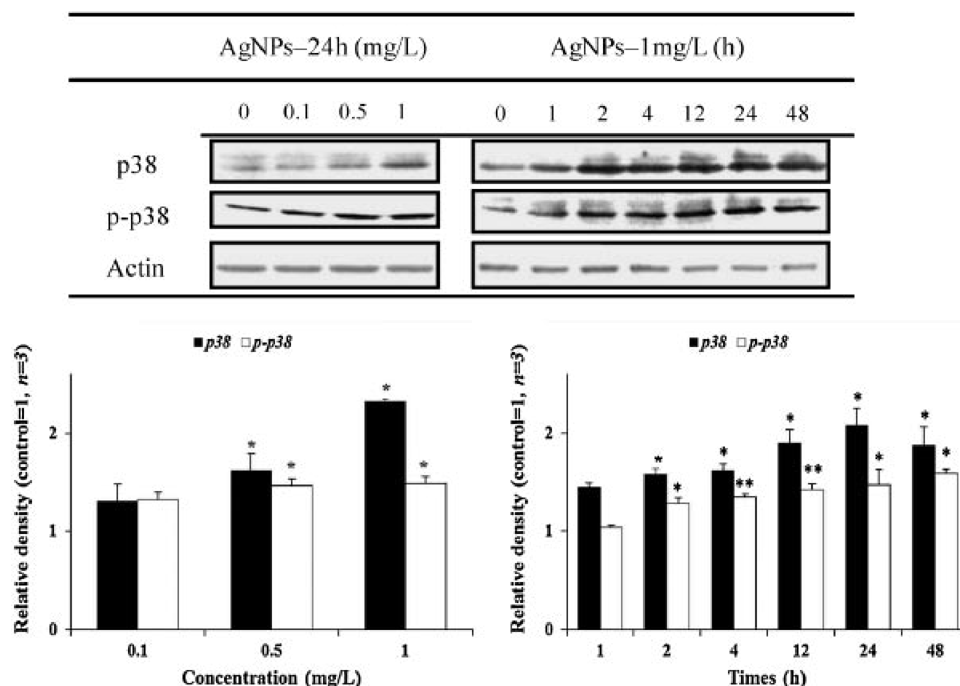

Fig. 2. The expressions of p38 and phospho-p38 protein (p-p38) in *wildtype (N2)* exposed to 0.1, 0.5, and 1 mg/L of silver nanoparticles (AgNPs) for 24 h, and 1 mg/L of AgNPs 1, 2, 4, 12, 24, and 48 h. Densitometric values of the expression of PMK-1 p38 mitogen-activated protein kinase were normalized using actin. The data represent the mean $\pm$ standard error of the mean of three individual experiments (control = 1, replicates $n = 3$; mean $\pm$ standard error of the mean; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the control).

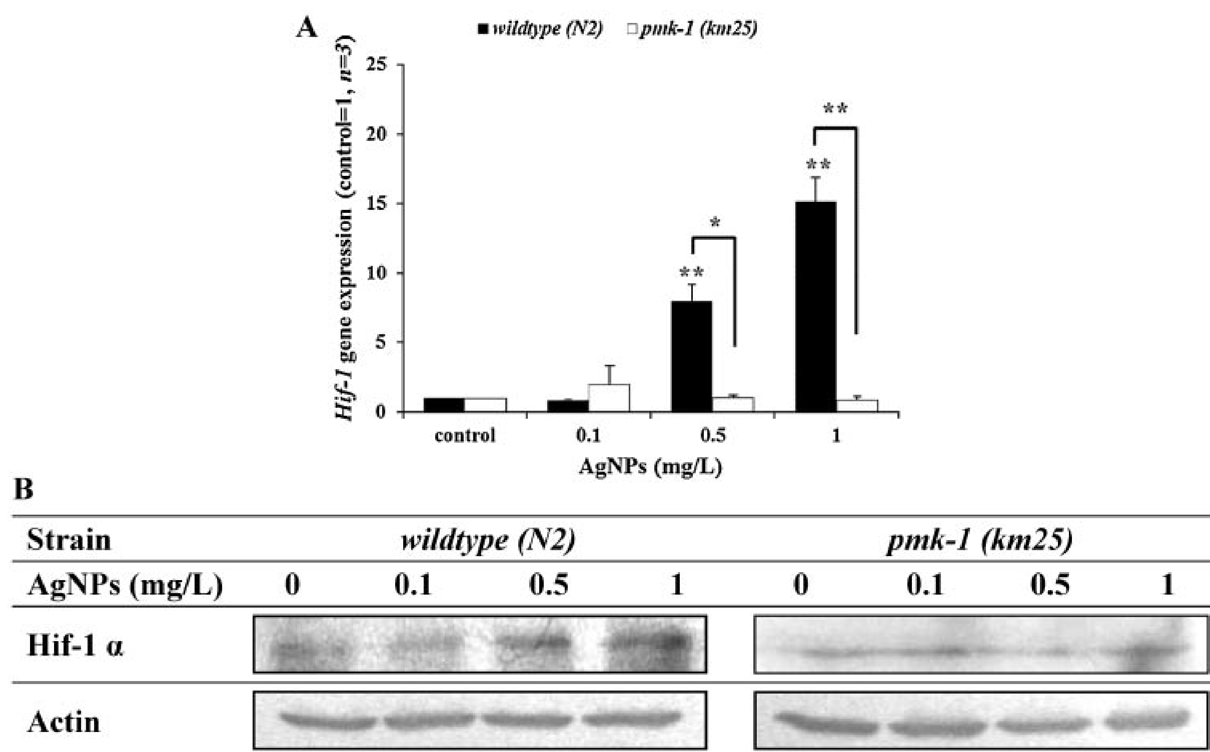

Fig. 4. The expressions of the *hif-1* gene (**A**) and HIF-1 protein (Hif-1 α) (**B**) in *wildtype (N2)* and *pmk-1 (km25)* mutant. Worms were exposed to 0.1, 0.5, and 1 mg/L of silver nanoparticles (AgNPs) for 24 h. Quantitative real time-polymerase chain reaction was performed for a gene expression analysis. The results are expressed as the mean value compared to the control (control = 1; $n = 3$; mean ± standard error of the mean using a one-way analysis of variance test [$^{*}p < 0.05$, $^{**}p < 0.01$]).

ionic silver partially contributed to the toxicity of AgNPs, but their underlying mechanisms seemed to be different, because AgNPs and $AgNO_3$ affect both reproduction and gene expression of the worm, but different groups of genes were affected. The present study supports this suggestion, because $AgNO_3$ did not induce a significant increase in ROS formation, whereas AgNPs did (Supplemental Data, Fig. S4). Moreover, the response of the *pmk-1 (km25)* mutant, in terms of reproduction and ROS formation, was no different from the response of *wildtype (N2)* to $AgNO_3$ exposure, whereas its response to AgNP exposure was significantly different. Further investigations are warranted to observe whether the toxicity is due to nano-specific AgNPs or partially to Ag ions. In this regard, in our previous study, we conducted Ag dissolution experiments on the same AgNPs that we used in this study, and found that the dissolution of AgNPs in water samples was very low [20]. Other groups also reported high stability and low dissolution rates of AgNPs in water [21,22]. They also reported negative correlations between dissolution of AgNPs and toxicity in zebrafish (*Danio rerio*) and *Daphnia* studies. Taking into account our previous experiment and studies of other groups, we assume that dissolution of Ag ion may not be the major factor of toxicity from AgNPs. It is also interesting to note that the presence of high concentration of chloride in the test media (K media) facilitates precipitation of Ag ions by forming AgCl, which, in turn, reduces the toxicity from Ag ions. This may explain the low toxicity of $AgNO_3$ and also suggests that the toxicity observed from AgNPs would be largely dependent on the nano-specific effect.

The fact that decreased reproductive potential and increased ROS formation induced by AgNPs in *wildtype (N2) C. elegans* were rescued in the *pmk-1 (km25)* mutant suggests the importance of the PMK-1 p38 MAPK in AgNP toxicity (Supplemental Data, Fig. S4A). Increased expressions of the *pmk-1* gene and p38 protein seem to be the defense mechanism of the worm against AgNP exposure (Figs. 1 and 2). It is known that on bacterial infection, *C. elegans* produces ROS as a host defense mechanism, which subsequently triggers stress-response mechanisms [23,24]; therefore, it cannot be ruled out that ROS increased in *wildtyp*e *(N2)* after exposure to AgNPs may not be produced directly by AgNPs but by *C. elegans*, as an immune response-like reaction to AgNP exposure, which may be compromised in *pmk-1 (km25)* mutant. Nevertheless, this hypothesis does not readily explain why ROS formation was observed only in connection with AgNPs but not with $AgNO_3$, because worms produce ROS as a nonspecific defense reaction. PMK-1 p38 MAPK plays a key role in the innate immunity of *C. elegans* [7,8]; however, little is known about whether PMK-1 p38 MAPK is involved in ROS production in the immune response of *C. elegans*, and even less about its involvement in the response to chemical exposure. Further study is ongoing in our lab to understand the differences or similarities between the roles of PMK-1 p38 MAPK in chemical response and immune response.

The signaling pathway of the activation of p38, nuclear factor-erythroid 2-related factor 2 (Nrf-2) and GST to oxidative stress have been well defined in mammalian systems [25]; therefore special attention was paid to the *skn-1* and GST as transcriptional and functional consequences of a signaling cascade of PMK-1 p38 MAPK. Inoue et al. [26] reported that the *C. elegans* PMK-1 p38 MAPK pathway regulates oxidative stress response through *skn-1* when arsenate was used as a chemical stressor. However, in our study, *skn-1* gene expression showed no statistical difference on exposure to AgNPs in

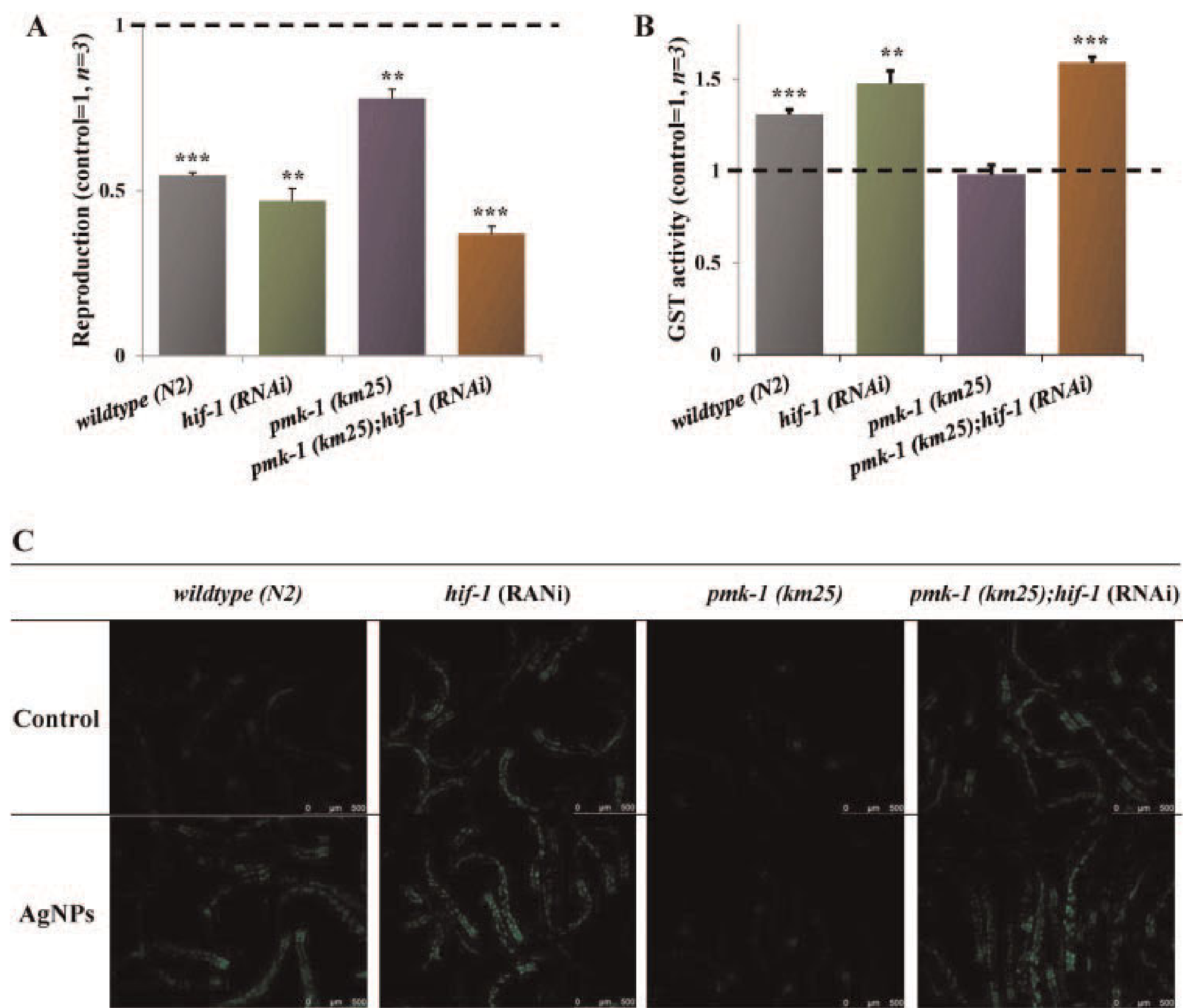

Fig. 5. Reproduction (**A**), glutathione *S*-transferase (GST) enzyme activity (**B**), and reactive oxygen species (ROS) formation (**C**) were measured in *wildtype (N2)* and the *pmk-1 (km25)* mutant of knockout *hif-1* gene using RNA interference (RNAi). Reproductive potential was investigated in 0.1 mg/L of silver nanoparticle (AgNP)-exposed *Caenorhabditis elegans (C. elegans)* for 72 h. GST enzyme activity were measured in 5 mg/L of AgNP-exposed *C. elegans* for 24 h. Results are expressed as the mean value compared to the control (control = 1, $n = 3$; mean ± standard error of the mean using a one-way analysis of variance test [$^{**}p < 0.01$, $^{***}p < 0.001$]). The formation of ROS was observed in worms exposed to 1 mg/L of AgNPs for 24 h. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

wildtype (N2) C. elegans, although surprisingly, *hif-1* gene expression increased significantly after exposure to AgNPs. As a critical regulator of cellular and systemic responses to low oxygen levels, HIF-1 has been extremely well studied in mammalian models [27,28]. The hypoxia response pathway is believed to be evolutionarily conserved, and thus the response of *C. elegans* to hypoxia has also been widely studied [29,30]. Our study showed that HIF-1 gene and protein expression were induced by AgNPs in *wildtype (N2) C. elegans*, but not the *pmk-1 (km25)* mutant, suggesting that AgNP-induced *hif-1* gene expression would result in the functional activation of this TF and would also require PMK-1 p38 MAPK activation. This was in good agreement with the previous study conducted by Emerling et al. [31] on a mouse model, in which mouse embryonic fibroblasts reported to lack p38 MAPK failed to stabilize HIF-1 α under hypoxia and oxidant stress, suggesting the involvement of the p38 MAPK cascade in ROS-dependent hypoxic responses. Chemicals inducing HIF-1 activation have been studied using certain compounds, such as cobalt chloride, deferoxamine, sodium hyposulfite, and sodium sulfite [32,33]. Most of these studies were conducted using hypoxia-mimicking agents for the purpose of establishing chemical hypoxia models, because physical hypoxia is technically difficult to control. However, few studies on the hypoxia signaling pathway have been conducted in toxicological context; to the best of our knowledge, none have been performed with NPs. Our results suggested that the hypoxia signaling pathway may be involved in the response to AgNPs in *C. elegans*. Further study may be needed to provide experimental evidence as to whether AgNPs may cause a hypoxic response in *C. elegans*.

The GSTs are a diverse family of multifunctional phase II detoxification enzymes, found in almost all living organisms, which play important roles in detoxification pathways and oxidative stress response. The GSTs have been widely used as a biomarker of ecotoxicity in various wildlife species [34,35]. In our previous study on the ecotoxicity of AgNPs with the aquatic midge (*Chironomus riparius*), GST mRNA expression was induced by a potent oxidative stress inducer, paraquat, which was also observed on exposure to AgNPs, suggesting the role of GST in the defense against oxidative stress and its potential application as a biomarker for AgNP exposure [36]. Our results suggested that the role of GST in the defense response to oxidative stress was also conserved in *C. elegans* and that its activation is PMK-1 p38 MAPK-dependent, but

HIF-1 independent; therefore, more detailed study on the roles of *C. elegans* GSTs in oxidative stress and their response towards NPs would be useful in understanding their roles in response to AgNP exposure.

Altered molecular/biochemical indicators identified as mechanisms of toxicity must be conclusively linked to adverse toxic outcomes. In this regard, it is interesting to note that ROS level and GST activity were observed to be negatively correlated with reproductive potential in AgNP-exposed worms. The *pmk-1 (km25)* mutant released very low levels of ROS on exposure to AgNPs, but showed more tolerant responses in their reproductive potential to AgNP exposure than the other three strains tested, that is, *wildtype (N2), hif-1* (RNAi), and *pmk-1 (km25);hif-1* (RNAi). This was only a speculation and thus does not provide direct evidence that decreased reproductive potential by AgNP exposure was due to ROS formation, but strongly suggests that oxidative stress contributed to altered reproduction, and that activation of PMK-1 p38 MAPK may be related to ROS formation, further affecting reproduction. To further investigate a direct relationship between PMK-1 and HIF-1 on AgNP toxicity, the response of *pmk-1-hif-1* double mutant, *pmk-1(km25)*;*hif-1* (RNAi) to AgNP exposure, was closely compared to that of *pmk-1* and *hif-1* single mutant, *pmk-1 (km25)* and *hif-1* (RNAi) (Fig. 5). Silver nanoparticle-induced toxicity *of pmk-1-hif-1* double mutant, *pmk-1(km25)*; *hif-1* (RNAi) was different from that produced by *pmk-1* single mutant, *pmk-1 (km25*); however, it was similar to *hif-1* single mutant, *hif-1* (RNAi). This result suggests that PMK-1 p38 MAPK functions upstream of HIF-1 in AgNP toxicity. The PMK-1 p38 MAPK and HIF-1 have been extensively studied in *C. elegans* stress response; however, to our knowledge, this is the first study investigated on their relationship in chemical response.

Finally, it is worth noting that *C. elegans* has been used not only as an ecotoxicity model, but also as a non-mammalian in vivo model for human toxicity screening. Therefore, it is important to identify how the mechanism of toxicity of AgNPs observed in studying *C. elegans* can be extrapolated to a mammalian system. This challenging task, which is not limited to nanotoxicity testing, may be achieved through comparative toxicity studies using various biological systems. For this reason, it was interesting to note that an increased level of p38 MAPK protein was also observed in human lymphoma cells following exposure to AgNPs [37] and in human bronchial cells following exposure to ceria NPs [17]. However, information is greatly lacking on the toxicity of AgNPs for any general correlation between the findings from *C. elegans* and in vitro and in vivo mammalian studies.

In conclusion, in the present study, to understand the mechanism of AgNP-induced altered reproduction in *C. elegans*, oxidative stress and the related signaling pathways were investigated. Exposure to silver nanoparticles was observed to lead to increased formation of ROS and activations of PMK-1, HIF-1, and GST, as well as decreased reproductive potential in *wild-type (N2) C. elegans*; in AgNP-exposed *pmk-1 (km25)* mutant however, ROS formation and HIF-1 and GST activation were not observed, and decreased reproductive potential was rescued. Overall, the results suggest that in *C. elegans*, oxidative stress is an important mechanism of AgNP-induced reproduction toxicity but not of $AgNO_3$, and PMK-1 p38 MAPK plays an important role in the response to oxidative stress induced by AgNPs. The results also suggest that HIF-1 and GST are involved in toxicity by AgNPs in a PMK-1 p38 MAPK-dependent manner.

SUPPLEMENTAL DATA

Figs. S1–S4.
Tables S1–S2. (308 KB PDF)

Acknowledgement—This work received financial support from the Mid-Career Researcher Program through a National Research Foundation of Korea grant funded by the Ministry of Education, Science and Technology (2011-0027489).

REFERENCES

1. Kahru A, Dubourguier HC. 2010. From ecotoxicology to nanoecotoxicology. *Toxicology* 269:105–119.
2. Oberdörster G, Stone V, Donaldson K. 2007. Toxicology of nanoparticles: A historical perspective. *Nanotoxicology* 1:2–25.
3. Wijnhoven SWP, Peijnenburg WJGM, Herberts CA, Hagens WI, Oomen AG, Heugens EHW, Roszek B, Bisschops J, Gosens I, Meent DVD, Dekkerks S, De jong WH, Zijverden MV, Sips AJAM, Geertsma RE. 2009. Nano-silver—A review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology* 3:109–138.
4. Miura N, Shinohara Y. 2009. Catatonic effect and apoptosis induction by silver nanoparticles in HeLa cells. *Biochem Biophys Res Commun* 390:733–737.
5. Roh JY, Sim SJ, Yi JH, Park KS, Chung KH, Ryu DY, Choi J. 2009. Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics. *Environ Sci Technol* 43: 3933–3940.
6. Kyriakis JM, Avruch J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81:807–869.
7. An JH, Vranas K, Lucke M, Inoue H, Hisamoto N, Matsumoto K, Blackwell TK. 2005. Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. *Proc Natl Acad Sci USA* 102:16275–16280.
8. Troemel ER, Chu SW, Reinke V, Lee SS, Ausubel FM, Kim DH. 2006. p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. *PLoS Genet* 2:e183.
9. Wang S, Wu L, Wang Y, Luo X, Lu Y. 2009. Copper-induced germline apoptosis in *Caenorhabditis elegans*: The independent roles of DNA damage response signaling and the dependent roles of MAPK cascades. *Chem Biol Interact* 180:151–157.
10. Shivers RP, Pagano DJ, Kooistra T, Richardson CE, Reddy KC, Whitney JK, Kamanzi O, Matsumoto K, Hisamoto N, Kim DH. 2010. Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in *Caenorhabditis elegans*. *PLoS Genet* 6:e1000892.
11. Brenner S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71–94.
12. Bae EJ, Lee JJ, Kim YH, Choi KH, Yi JH. 2010. Bacterial cytotoxicity of the silver nanoparticle related to physicochemical metrics and agglomeration properties. *Environ Toxicol Chem* 29:2154–2160.
13. Halliwell B, Gutteridge JMC. 2007. *Free Radicals in Biology and Medicine*, 4th ed. Oxford University Press, New York, NY, USA.
14. Eruslanov E, Kusmartsev S. 2010. Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods Mol Biol* 594:57–72.
15. Ates B, Abraham L, Ercal N. 2008. Antioxidant and free radical scavenging properties of N-acetylcysteine amide (NACA) and comparison with N-acetylcysteine (NAC). *Free Radic Res* 42:372–377.
16. Flora SJ. 2009. Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. *Oxid Med Cell Longev* 2:191–206.
17. Eom HJ, Choi J. 2009. Oxidative stress of CeO_2 nanoparticles via p38-Nrf-2 signaling pathway in human bronchial epithelial cell, Beas-2B. *Toxicol Lett* 187:77–83.
18. Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 249:7730–7739.
19. Roh JY, Park YK, Park KS, Choi J. 2010. Ecotoxicological investigation of CeO_2 and TiO_2 nanoparticles on the soil nematode *Caenorhabditis elegans* using gene expression, growth, fertility, and survival as endpoints. *Environ Toxicol Pharmacol* 29:167–172.
20. Nair PMG, Park SY, Lee SW, Choi J. 2010. Differential expression of the ribosomal protein gene, gonadotrophin releasing hormone gene and Balbiani ring protein gene as mechanisms of the toxicity on exposure to silver nanoparticles in fourth instar larvae of the aquatic midge, *Chironomus riparius*. *Aquat Toxicol (Amst)* 101:31–37.

21. Asharani PV, Lian WY, Gong Z, Valiyaveettil S. 2008. Toxicity of silver nanoparticles in zebrafish models. *Nanotechnology* 19:255102.
22. Griffitt RJ, Luo J, Gao J, Bonzongo JC, Barber DS. 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environ Toxicol Chem* 27:1972–1978.
23. Chávez V, Mohri-Shiomi A, Maadani A, Vega LA, Garsin DA. 2007. Oxidative stress enzymes are required for DAF-16-mediated immunity due to generation of reactive oxygen species by *Caenorhabditis elegans*. *Genetics* 176:1567–1577.
24. Chávez V, Mohri-Shiomi A, Garsin DA. 2009. Ce-Duox1/BLI-3 generates reactive oxygen species as a protective innate immune mechanism in *Caenorhabditis elegans*. *Infect Immun* 77:4983–4989.
25. Giudice A, Montella M. 2006. Activation of the Nrf2-ARE signaling pathway: A promising strategy in cancer prevention. *Bioessays* 28: 169–181.
26. Inoue H, Hisamoto N, An JH, Oliveira RP, Nishida E, Blackwell TK, Matsumoto K. 2005. The *C. elegans* p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. *Genes Dev* 19:2278–2283.
27. Maxwell PH. 2005. The HIF pathway in cancer. *Semin Cell Dev Biol* 16:523–530.
28. Wenger RH, Stiehl DP, Camenisch G. 2005. Integration of oxygen signaling at the consensus HRE. *Sci STKE* 306:re12.
29. Jiang H, Guo R, Powell-Coffman JA. 2001. The *Caenorhabditis elegans* hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc Natl Acad Sci USA* 98:7916–7921.
30. Powell-Coffman JA. 2010. Hypoxia signaling and resistance in *C. elegans*. *Trends Endocrinol Metab* 21:435–440.
31. Emerling BM, Platanias LC, Black E, Nebreda AR, Davis RJ, Chande NS. 2005. Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. *Mol Cell Biol* 25:4853–4862.
32. Grasselli F, Basini G, Bussolati S, Bianco F. 2005. Cobalt chloride, a hypoxia-mimicking agent, modulates redox status and functional parameters of cultured swine granulosa cells. *Reprod Fertil Dev* 17:715–720.
33. Jiang B, Ren C, Li Y, Lu Y, Li W, Wu Y, Gao Y, Ratcliffe PJ, Liu H, Zhang C. 2010. Sodium sulfite is a potential hypoxia inducer that mimics hypoxic stress in *Caenorhabditis elegans*. *J Biol Inorg Chem* 16: 267–274.
34. Tu HT, Silvestre F, Wang N, Thome JP, Phuong NT, Kestemont P. 2010. A multi-biomarker approach to assess the impact of farming systems on black tiger shrimp (*Penaeus monodon*). *Chemosphere* 81: 1204–1211.
35. Canesi L, Fabbri R, Gallo G, Vallotto D, Marcomini A, Pojana G. 2010. Biomarkers in *Mytilus galloprovincialis* exposed to suspensions of selected nanoparticles (Nano carbon black, C60 fullerene, Nano-TiO2, Nano-SiO2). *Aquat Toxicol (Amst)* 100:168–177.
36. Nair PMG, Choi J. 2011. Identification; characterization and expression profiles of *Chironomus riparius* glutathione *S*-transferase (GST) genes in response to cadmium and silver nanoparticles exposure. *Aquat Toxicol (Amst)* 101:550–560.
37. Eom HJ, Choi J. 2010. p38 MAPK activation, DNA damage, cell cycle arrest and apoptosis as mechanisms of toxicity of silver nanoparticles in Jurkat T cells. *Environ Sci Technol* 44:8337–8342.