Ecotoxicology and Environmental Safety 163 (2018) 456–464

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Functional disruption in epidermal barrier enhances toxicity and accumulation of graphene oxide

Xuecheng Ding[a,b], Qi Rui[a,*], Dayong Wang[b,*]

[a] College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China
[b] Key Laboratory of Environmental Medicine Engineering in Ministry of Education, Medical School, Southeast University, Nanjing 210009, China

ARTICLE INFO

Keywords:
Epidermal barrier
Molecular basis
Nanotoxicity
Graphene oxide
Caenorhabditis elegans

ABSTRACT

In *Caenorhabditis elegans*, mutation of *mlt-7* causes the deficits in epidermal barrier. Using the nematodes with epidermal-specific RNA interference (RNAi) knockdown of *mlt-7* as a genetic tool, we found that epidermal-specific RNAi knockdown of *mlt-7* resulted in a susceptibility to graphene oxide (GO) toxicity, and enhanced GO accumulation in the body. Epidermal-development related proteins of BLI-1 and IFB-1 acted as downstream targets of MLT-7, and mediated the function of MLT-7 in maintaining the epidermal barrier. Antimicrobial proteins of NLP-30 and CNC-2 also acted as downstream targets of MLT-7 in the regulation of GO toxicity. Epidermal-specific RNAi knockdown of *nlp-30* or *cnc-2* enhanced GO toxicity and accumulation in *bli-1(RNAi)* or *ifb-1(RNAi)* nematodes. Our data highlights the importance of maintaining normal epidermal barrier for nematodes against the GO toxicity.

1. Introduction

Graphene and its derivatives constitute a novel class of carbon-based engineered nanomaterials (ENMs) (Geim, 2009). Graphene and its derivatives, such as graphene oxide (GO), possess the properties of large surface area, high thermal conduction, chemical stability, and amphipathicity (Geim, 2009). GO can be potentially at least used in drug delivery, biosensor, and environmental remediation, and etc (Geim, 2009; Bitounis et al., 2013; Zhu et al., 2016; Cheng and Wang, 2016). Due to the potential to overtake carbon nanotubes in the applications (Geim and Novoselov, 2007), a large amount of graphene ENMs, including the GO, are possibly released into the environment in the future.

Now, some evidence has been raised to demonstrate the cytotoxicity of GO, such as cell division inhibition, apoptosis, and mutagenicity (Liu et al., 2016a, 2016b; Chang et al., 2011; Duan et al., 2015; Qu et al., 2013). The pulmonary toxicity, neurotoxicity, and reproductive toxicity of GO were detected in mammals or zebrafish (Yang et al., 2013; Li et al., 2013; Liang et al., 2015; Ren et al., 2016). *Caenorhabditis elegans*, a non-mammalian animal model (Brenner, 1974), has been widely applied in toxicological study of different toxicants due to its sensitivity to environmental exposure (Chen et al., 2013; Leung et al., 2008; Khare et al., 2015; Cong et al., 2015; Shakoor et al., 2016; Zhuang et al., 2016; Yang et al., 2016b; Maurer et al., 2016; O'Donnell et al., 2017; Li et al., 2017, 2018; Zhao et al., 2017a; Gonzalez-Moragas et al., 2017; Xiao et al., 2018b; Dong et al., 2018). In nematodes, GO exposure could cause toxicity on the functions of both primary (intestine) and secondary (neurons and reproductive organs) targeted organs (Zhang et al., 2012; Chatterjee et al., 2015, 2017; Yang et al., 2016a; Jung et al., 2015; Xiao et al., 2017, 2018a; Li et al., 2017; Chen et al., 2017).

In organisms, bioavailability is a crucial cellular contributor to GO toxicity formation (Li et al., 2013; Zhang et al., 2012; Yang et al., 2013). In mammals, GO could be accumulated in different organs, such as lung, liver, and kidney (Yang et al., 2013; Li et al., 2013). In nematodes, GO could be accumulated in various organs, including intestinal cells, gonad, and spermatheca (Ren et al., 2018). Moreover, it has been proven for the importance of intestinal barrier against toxicity and translocation of ENMs in nematodes (Ding et al., 2018; Qu et al., 2018).

The nematode epidermis is a protective collagenous extracellular matrix, and this matrix is synthesized by the underlying hypodermis surrounding the body (Johnstone, 2000). Epidermal barrier is another important biological barrier for nematodes against the environmental toxicants or stresses (Page and Johnstone, 2007). So far, the molecular basis for epidermal barrier against the toxicity of ENMs is still largely unclear. *mlt-7* encoding a peroxidase expressed in epidermis (Moribe et al., 2012). MLT-7 is required for the proper epidermal molting and re-synthesis (Thein et al., 2009). Mutation of *mlt-7* would result in the hypermeability to dye of Hoechst 33258, implying the formation of

* Corresponding authors.
E-mail addresses: ruiqi@njau.edu.cn (Q. Rui), dayongw@seu.edu.cn (D. Wang).

https://doi.org/10.1016/j.ecoenv.2018.07.102
Received 3 July 2018; Received in revised form 22 July 2018; Accepted 24 July 2018

impaired function of epidermal barrier (Thein et al., 2009). In this study, using the nematodes with epidermal-specific RNA interference (RNAi) knockdown of *mlt-7* as a genetic tool, we investigated the molecular basis for epidermal barrier against GO toxicity. BLI-1 and IFB-1 are two hypodermal proteins (Karabinos et al., 2003; Jackson et al., 2014). *bli-1* encodes a cuticular collagen, and *ifb-1* encodes an intermediate filament protein. BLI-1 and IFB-1 were identified as downstream targets for epidermal MLT-7 in the regulation of GO toxicity. Our data highlight the crucial function of epidermal barrier for nematodes against the toxicity of environmental ENMs. Our data provide the clues for further understanding the molecular basis of epidermal barrier against the translocation and the toxicity of environmental toxicants.

2. Materials and methods

2.1. GO preparation and properties

We prepared GO using the natural graphite (Kovtyukhova et al., 1999). GO thickness was approximately 1.0 nm based on atomic force microscopy analysis (Fig. S1A). Sizes for most of GO particles after sonication (40 kHz, 100 W, 30 min) were in the range of 40–50 nm (Fig. S1B). GO sheet had a D band (1335 cm^{-1}) and a G band (1595 cm^{-1}), respectively, based on Raman spectroscopy analysis (Fig. S1C). Zeta potential of GO (10 mg/L) was -22.5 ± 2.3 mV based on the measurement by dynamic light scattering (DLS).

2.2. Strains and culture

The used strains include wild-type (N2), and transgenic strains of NR222/*rde-1(ne219);kzIs9* and *Is(Pmlt-7-mlt-7::GFP)*. NR222 was used a tool for epidermal-specific RNAi of certain genes (Qadota et al., 2007). Nematodes were maintained normally as described (Brenner, 1974), and *Escherichia coli* OP50 was used as a food source. To prepare the age synchronous L1-larvae or L2-larvae populations, the adult hermaphrodites were lysed using a bleaching mixture (0.45 M NaOH, 2% HOCl) in order to collect the eggs.

2.3. Exposure and endpoints

GO stock solution (1 mg/mL) was prepared in K medium by sonication (40 kHz, 100 W, 30 min). Previous study has indicated that exposure to GO ($\geq$ 1 mg/L) caused induction of intestinal reactive oxygen species (ROS) production, reduction in brood size, and suppression in locomotion behavior (Wu et al., 2013). In this study, 10 mg/L was used as working concentration, which was diluted from the GO stock solution using the K medium. GO exposure was performed from L1-larvae to adult day-1 in liquid with the addition of OP50, or from L4 larvae for 48 h with the addition of OP50.

ROS production is an endpoint to reflect the state of intestinal function (Qu et al., 2017). The method was performed as described (Ren et al., 2017; Zhao et al., 2017b). Nematodes were incubated with 5′,6′-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA, 1 μM) for 3 h in darkness. After that, the animals were examined under the condition of certain excitation wavelength (488 nm) and emission filter (510 nm) under a laser scanning confocal microscope. Relative fluorescence intensity of intestinal ROS signals was semi-quantified in comparison to intestinal autofluorescence. Before taking the pictures, the NaN_3 (0.1%) was added to stop the movement of nematodes.

2.4. GO distribution and accumulation

GO/Rhodamine B (GO/Rho B) was prepared by mixing 0.3 mL Rho B (1 mg/mL) with 5 mL GO suspension (0.1 mg/mL) as described (Ding et al., 2018). Dialysis against distilled water (over 72 h) was performed to remove the unbound Rho B. GO/Rho B exposure was performed from L4 larvae for 48-h. Nematodes were analyzed under a laser scanning confocal microscope. Before taking the pictures, the NaN_3 (0.1%) was added to stop the movement of nematodes.

2.5. Reverse-transcription and quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNAs extracted from nematodes using an RNeasy Mini kit, were reverse transcribed using a PrimeScript ™ RT reagent kit. SYBR Premix Ex Taq™ was used for qRT-PCR in order to amplify certain gene products. For the examined genes, relative quantification was performed in comparison to reference gene (*tba-1* encoding a tubulin), and expressed as the comparison ratio. The experiments were conducted in triplicate. Primer information is provided (Table S1).

2.6. DNA constructs and germline transformation

mlt-7 promoter was amplified from wild-type N2 DNA, and cloned into an empty vector (pPD95_77). *mlt-7* cDNA was further subcloned into pPD95_77 behind the *mlt-7* promoter. Germline transformation was conducted by coinjecting both testing DNA (10–40 μg/mL) and marker DNA of *unc-119(+)* (60 μg/mL) into nematode gonads as described (Mello and Fire, 1995). Primer information is provided (Table S2).

2.7. RNAi

In order perform the RNAi experiments, *E. coli* HT115 (DE3) expressing double-stranded RNA with the homologous to the examined gene(s) was fed to the animals as described (Wu et al., 2016). L2 larvae were cultured on RNAi plates for 1 day until the animals develop into the L4 larvae. L4 larvae were further exposed to GO or GO/RhoB. RNAi efficiency was confirmed by qRT-PCR (Fig. S2).

2.8. Statistical analysis

Statistical analysis was conducted using the SPSS 12.0 software. Differences between the groups were analyzed using analysis of variance (ANOVA).

3. Results

3.1. Effect of GO exposure on MLT-7 expression

After prolonged exposure, GO (10 mg/L) could not significantly influence transcriptional expression of *mlt-7* (Fig. S3A). GO exposure (100 mg/L or 1 g/L) also could not affect transcriptional expression of *mlt-7* (data not shown). To further investigate the effect of GO exposure on MLT-7 expression, we constructed the MLT-7::GFP transgenic strain. MLT-7 is expressed in the nuclei of hypodermal cells in adult nematodes (Thein et al., 2009). GO exposure (10 mg/L) could not affect the expression level, as well as the expression pattern, of MLT-7::GFP (Fig. S3B).

3.2. GO toxicity in nematodes with epidermal-specific RNAi knockdown of mlt-7

Again, we investigated the GO toxicity in nematodes with epidermal-specific RNAi knockdown of *mlt-7*. In NR222 strain, we could not observe the induction of intestinal ROS production (Fig. 1). After the GO exposure, we observed the significant induction of intestinal ROS production in NR222 strain (Fig. 1). Under the normal conditions, epidermal-specific RNAi knockdown of *mlt-7* also did not induce induction of intestinal ROS production (Fig. 1). In contrast, after the GO exposure, we detected the more significant induction of intestinal ROS production in *mlt-7(RNAi)* nematodes compared with NR222 (Fig. 1). Therefore, epidermal-specific RNAi knockdown of *mlt-7* may result in a