Dysregulation of Neuronal Gαo Signaling by Graphene Oxide in Nematode Caenorhabditis elegans

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Exposure to graphene oxide (GO) induced some dysregulated microRNAs (miRNAs), such as the increase in mir-247, in nematode Caenorhabditis elegans. We here further identified goa-1 encoding a Gαo and pck-1 encoding a serine/threonine protein kinase as the targets of neuronal mir-247 in the regulation of GO toxicity. GO exposure increased the expressions of both GOA-1 and PKC-1. Mutation of goa-1 or pck-1 induced a susceptibility to GO toxicity, and suppressed the resistance of mir-247 mutant to GO toxicity. GOA-1 and PKC-1 could also act in the neurons to regulate the GO toxicity, and neuronal overexpression of mir-247 could not affect the resistance of nematodes overexpressing neuronal goa-1 or pck-1 lacking 3′-UTR to GO toxicity. In the neurons, GOA-1 acted upstream of diacylglycerol kinase/DGK-1 and PKC-1 to regulate the GO toxicity. Moreover, DGK-1 and GOA-1 functioned synergistically in the regulation of GO toxicity. Our results highlight the crucial role of neuronal Gαo signaling in response to GO in nematodes.

Short noncoding microRNAs (miRNAs) exist in many organisms, including human. miRNAs usually regulate various biological processes by suppressing expression and function of certain targeted genes post-transcriptionally. The founding members of miRNA family, such as lin-4 and let-7, were firstly identified in Caenorhabditis elegans via forward genetic screen. Due to sensitivity to environmental toxicants or stresses, C. elegans has been widely used in toxicity assessment and toxicological study of various engineered nanomaterials (ENMs). Moreover, certain amount of miRNAs in response to ENMs, such as carbon-based ENMs, have been identified in nematodes. Graphene oxide (GO), a member of graphenic nanomaterials, can be potentially applied in at least drug delivery, biosensors, bioimaging, cancer therapy, catalytic, and environmental decontamination due to its unique physical and chemical properties. With the increase in these potential applications, availability of GO to both human and environmental organisms has received great attention recently. Some in vitro and in vivo studies have demonstrated the cytotoxicity, the pulmonary toxicity, and reproductive toxicity of GO. In nematodes, exposure to GO resulted in damage on the functions of both primary targeted organs (such as intestine) and secondary targeted organs (such as neurons and reproductive organs). Among the dysregulated miRNAs in GO exposed nematodes, mir-247 is the homologous of human miR-134 and miR-708, and the expression of mir-247 was up-regulated by GO exposure. Mutation of mir-247 induced a resistance to GO toxicity. mir-247 could act in the neurons to regulate the GO toxicity, and neuronal overexpression of mir-247 resulted in a susceptibility to GO toxicity. However, molecular mechanism for the role of neuronal mir-247 in the regulation of GO toxicity is still unclear. Therefore, we here further employed C. elegans to investigate the underlying mechanism for the function of neuronal mir-247 in regulating GO toxicity. We here identified GOA-1, an ortholog of heterotrimeric G protein α subunit Gαo, as a downstream target for neuronal mir-247 in regulating GO toxicity. Moreover, in the neurons, a signaling cascade of GOA-1-DGK-1/PKC-1 was raised to explain the underlying mechanism for induction of GO toxicity. Our study provides an important molecular basis for neuronal Gαo signaling in response to GO in organisms.

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Results

GOA-1, PKC-1, and CEH-18 might act as the possible targets for neuronal mir-247 in the regulation of GO toxicity. We employed TargetScan software (http://www.targetscan.org/worm_52/) with preferentially conserved targeting (PCT) between 0 and 1 and miRBase (http://www.mirbase.org) with a score threshold of −0.1 to predict potential targets for mir-247 in the regulation of GO toxicity by searching for the presence of conserved sites that match seed region of mir-247. Among the searched genes possibly acting as the targets for mir-247, goa-1, gpb-2, tcer-1, eat-16, rps-8, lin-41, cwn-1, vhp-1, rab-39, ceh-18, acy-1, daf-19, pkc-1, lrk-1, cnb-1, dve-1, acs-22, egl-15, kin-1, mdt-15, apr-1, and dkf-2 are associated with the regulation of stress response in nematodes. The anticipated phenotype of nematodes with mutation of targeted gene is susceptible to GO toxicity. However, mutation of cwn-1 or apr-1 was resistant to GO toxicity.

Our previous study has suggested that mir-247 acts in the neurons to regulate GO toxicity. Among the other 20 possible targeted genes of mir-247, goa-1, gpb-2, tcer-1, eat-16, rps-8, lin-41, vhp-1, rab-39, ceh-18, acy-1, daf-19, pkc-1, lrk-1, cnb-1, dve-1, acs-22, egl-15, kin-1, mdt-15, and dkf-2 can be expressed in the neurons (http://www.wormbase.org/species/c_elegans/gene). The working concentration (10 mg/L) was selected as described previously. Prolonged exposure to GO (10 mg/L) did not affect expressions of gpb-2, tcer-1, lin-41, vhp-1, rab-39, acy-1, daf-19, pkc-1, lrk-1, cnb-1, dve-1, acs-22, egl-15, kin-1, mdt-15, and dkf-2 (Fig. S1a). In contrast, GO (10 mg/L) decreased expressions of goa-1, eat-16, ceh-18, and pkc-1 and increased the transcriptional expression of dev-1 (Fig. S1a). Since GO exposure could increase the mir-247 expression, the anticipated expression tendency of targeted genes after GO exposure should be suppression in wild-type nematodes.

We next examined the expressions of goa-1, eat-16, ceh-18, and pkc-1 in GO exposed nematodes overexpressing the neuronal mir-247. Neuronal overexpression of mir-247 could not obviously affect the transcriptional expression of eat-16 after GO (10 mg/L) exposure (Fig. S1b). In contrast, neuronal overexpression of mir-247 could significantly suppress the transcriptional expressions of goa-1, ceh-18, and pkc-1 after GO (10 mg/L) exposure (Fig. S1b). Therefore, goa-1, ceh-18, and pkc-1 might be the targeted genes for neuronal mir-247 in the regulation of GO toxicity in C. elegans, goa-1 encodes an ortholog of the heterotrimeric G protein α subunit, ceh-18 encodes a POU-class homeodomain transcription factor, and pkc-1 encodes a serine/threonine protein kinase.
Mutation of **goa-1** or **pkc-1** induced a susceptibility to GO toxicity. Using the **goa-1**(sa734), **ceh-18**(ok1082), and **pkc-1**(ok563) mutants, we next investigated the possible function of GOA-1, CEH-18, and PKC-1 in the regulation of GO toxicity. Under the normal conditions, mutation of **goa-1**, **ceh-18**, or **pkc-1** did not induce the intestinal ROS production and affect the locomotion behavior (Fig. S2). In nematodes, mutation of **ceh-18** did not influence the toxicity of GO (10 mg/L) (Fig. S2). In contrast, mutation of **goa-1** or **pkc-1** resulted in a susceptibility to GO toxicity (Fig. S2). Therefore, both GOA-1 and PKC-1 may negatively regulate the GO toxicity.

Genetic interaction between **mir-247** and **goa-1** or **pkc-1** in the regulation of GO toxicity. To confirm the molecular interaction between **mir-247** and **goa-1** in the regulation of GO toxicity, we compared the GO toxicity in **goa-1**(RNAi);**mir-247**(n4505) with that in **goa-1**(RNAi) (**Fig. 1**). We also compared the GO toxicity in **pkc-1**(RNAi);**mir-247**(n4505) with that in **pkc-1**(RNAi) (**Fig. 1**). Similarly, after GO exposure, the toxicity in **pkc-1**(RNAi);**mir-247**(n4505) was similar to those in **pkc-1**(RNAi) (**Fig. 1**). Therefore, the neuronal **mir-247** may suppress the function of both GOA-1 and PKC-1 in the regulation of GO toxicity.

Neuronal overexpression of **mir-247** could not affect the resistance of nematodes overexpressing neuronal **goa-1** or **pkc-1** lacking 3′-UTR to GO toxicity. To further confirm the roles of GOA-1 and PKC-1 as the target of neuronal **mir-247**, we next investigated the genetic interaction between **mir-247** and **goa-1** or **pkc-1** in the neurons to regulate the GO toxicity. We introduced the **goa-1** or **pkc-1** lacking 3′-UTR driven by **unc-14** promoter into the nematodes overexpressing neuronal **mir-247**. After GO exposure, the transgenic strain **Is(Punc-14-goa-1-3′-UTR);Ex(Punc-14-mir-247)** exhibited the similar resistance to GO toxicity to that in the transgenic strain **Is(Punc-14-goa-1-3′-UTR)** (**Fig. 2**). Additionally, the transgenic strain **Is(Punc-14-pkc-1-3′-UTR);Ex(Punc-14-mir-247)** showed the similar resistance to GO toxicity to that in the transgenic strain **Is(Punc-14-pkc-1-3′-UTR)** (**Fig. 2**).
Tissue-specific activity of *goa-1* in the regulation of GO toxicity. *goa-1* gene is expressed in the pharynx, the neurons, and the muscle. PKC-1 is only expressed in the neurons. Using tissue-specific promoters, we investigated the tissue-specific activity of *goa-1* in the regulation of GO toxicity. Rescue assay by expression of *goa-1* in the pharynx or the muscle did not significantly influence the susceptibility of *goa-1*(sa734) mutant to GO toxicity (Fig. 3). Different from these, expression of *goa-1* in the neurons could significantly suppress the susceptibility of *goa-1*(sa734) mutant to GO toxicity (Fig. 3). These results suggest that both GOA-1 and PKC-1 may act in the neurons to regulate the GO toxicity.

Identification of downstream targets for GOA-1 in the Gαo signaling pathway in the regulation of GO toxicity. In the Gαo signaling pathway, DGK-1 is a downstream target for GOA-1, and *dgk-1* encodes an ortholog of mammalian diacylglycerol kinase theta (DGKθ)45. In GO (10 mg/L) exposed *goa-1* mutant, we detected the significant decrease in expressions of both *pkc-1* and *dgk-1* compared with those in GO (10 mg/L) exposed wild-type nematodes (Fig. 4a), which implies that both PKC-1 and DGK-1 may act as important downstream targets for GOA-1 during the control of GO toxicity.

Using induction of intestinal ROS production and locomotion behavior as the toxicity assessment endpoints, we found that the *dgk-1*(sy428) mutant was susceptible to GO toxicity (Fig. 4b,c), suggesting that GOA-1 may positively regulate GO toxicity by affecting functions of PKC-1 and DGK-1.

Genetic interaction between GOA-1 and PKC-1 or DGK-1 in the regulation of GO toxicity. To determine the genetic interaction between *goa-1* and *dgk-1* or *pkc-1* in the regulation of GO toxicity, we examined the effects of mutation of *dgk-1* or *pkc-1* on GO toxicity in transgenic strain overexpressing the neuronal *goa-1*. The nematodes overexpressing neuronal *goa-1* exhibited the resistance to GO toxicity (Fig. 5). In contrast, after the GO exposure, *dgk-1* or *pkc-1* mutation suppressed the resistance of nematodes overexpressing neuronal *goa-1* to GO toxicity (Fig. 5). Therefore, neuronal GOA-1 may act upstream of both DGK-1 and PKC-1 to regulate the GO toxicity.
Genetic interaction between PKC-1 and DGK-1 in the regulation of GO toxicity. We further investigated the genetic interaction between the PKC-1 and DGK-1. After GO exposure, we observed the more severe GO toxicity in double mutant of pkc-1(ok563);dgk-1(sy428) compared with that in single mutant of pkc-1(ok563) or dgk-1(sy428) (Fig. 6a,b).

Discussion
In this study, we first provide several lines of evidence to indicate the potential role of GOA-1 and PKC-1 as the targets for neuronal mir-247 in the regulation of GO toxicity. First of all, expressions of both GOA-1 and PKC-1 could be suppressed by GO exposure, and their expressions could be further decreased by overexpression of neuronal mir-247 in GO exposed nematodes (Fig. S1). Secondly, in nematodes, the phenotypes in GO exposed goa-1(sa734) or pkc-1(ok563) mutant were opposite to those in GO exposed mir-247/797(n4505) mutant (Fig. S2). Thirdly, we found that mutation of goa-1 or pkc-1 could effectively inhibit the resistance of mir-247/797(n4505) mutant to GO toxicity (Fig. 1). Moreover importantly, we observed that neuronal overexpression of mir-247 did not influence the resistance of transgenic strain overexpressing neuronal goa-1 lacking 3′-UTR or pkc-1 lacking 3′-UTR to GO toxicity (Fig. 2), implying the binding of mir-247 to the 3′-UTR of goa-1 or pkc-1. Previous study has identified the EGL-5 as the target for mir-247 in the control of male tail development46. In this study, we identified the GOA-1 and the PKC-1 as the potential targets for mir-247 during the control of GO toxicity formation in hermaphrodite nematodes.

GOA-1 activity is required for the regulation of asymmetric cell division in the early embryo, innate immunity, olfactory-mediated behaviors, and decision-making42,43,47,48. In this study, we further found the novel function of Gαo signaling in the control of nanotoxicity. In C. elegans, goa-1 mutation induced a susceptibility of nematodes to GO toxicity (Fig. 1), implying that goa-1-encoded Gαo signaling negatively regulates GO toxicity.

The tissue-specific activity assays indicated that the neuronal GOA-1 regulates the GO toxicity (Fig. 3). In organisms, G protein coupled receptors (GPCRs), seven-transmembrane receptors, can sense the environmental signals or molecules outside the cell and activate the inside signal transduction pathways and, ultimately, the cellular responses by coupling with the G proteins49. The function of goa-1-encoded Gαo signaling in the neurons...
implies that certain GPCRs in the neurons may be activated or suppressed by GO exposure, and the affected neuronal GPCRs may further function through the goa-1/Gαo-mediated signaling cascade to regulate the GO toxicity.

In this study, GOA-1 could further act upstream of diacylglycerol kinase/DGK-1 and PKC-1 to regulate the GO toxicity. Under the condition of GO exposure, goa-1 mutation decreased dgk-1 and pkc-1 expressions (Fig. 4a). Additionally, dgk-1 or pkc-1 mutation inhibited the resistance of transgenic strain overexpressing neuronal goa-1 to GO toxicity (Fig. 5). Therefore, a corresponding signaling cascade of GOA-1-DGK-1/PKC-1 can be raised to explain the molecular basis for neuronal mir-247 in response to GO exposure (Fig. 6c).

Prolonged exposure to GO (≥10 μg/L) increased the mir-247 expression. Meanwhile, neuronal overexpression of mir-247 induced a susceptibility to GO toxicity. Therefore, the raised neuronal signaling cascade of mir-247-GOA-1-DGK-1/PKC-1 provides an important molecular mechanism for the potential GO toxicity induction in nematodes.

In this study, we further found that DGK-1 and PKC-1 functioned synergistically to regulate GO toxicity (Fig. 6a,b). PKC-1 plays a role in regulating function of nervous system, such as the neurotransmission. This observation implies the possibility that, besides the normally considered downstream diacylglycerol kinase/DGK-1 signaling, the neuronal GOA-1/Gαo signaling may also regulate the GO toxicity by influencing the neurotransmission process. Our previous study has identified the NLG-1-PKC-1 signaling cascade in the regulation of GO toxicity. Our results suggest that PKC-1 may act as an important link between the Gαo/GOA-1 signaling and the NLG-1 signaling in the regulation of GO toxicity. Additionally, PKC-1 may further act as the direct target for mir-247 in the regulation of GO toxicity (Fig. 2). These results imply the potential crucial role of neurotransmission process in the toxicity induction in GO exposed nematodes.

In conclusion, we found that Gαo/GOA-1 and PKC-1 functioned as targets for neuronal mir-247 in the regulation of GO toxicity. GOA-1 further acted upstream of both diacylglycerol kinase/DGK-1 signaling and PKC-1 signaling to regulate the GO toxicity. During the control of GO toxicity, DGK-1 and PKC-1 acted in parallel signaling pathways. Our data provide the important molecular basis for neuronal Gαo signaling in response to GO. Additionally, our results imply that certain neuronal GPCRs may sense the GO exposure, and the affected neuronal GPCRs may further function through the goa-1/Gαo-mediated signaling cascade to regulate the GO toxicity.
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Methods
Preparation and characterization of GO. GO was prepared from natural graphite powder using a modified Hummer’s method. The analysis of atomic force microscopy (AFM, SPM-9600, Shimadzu, Japan) indicated that the thickness of GO was approximately 1.0 nm in topographic height, corresponding to the property of one layer. Sizes of most of the GO in K-medium after sonication (40 kHz, 100 W, 30 min) were in the range of 40–50 nm. Assay on the Raman spectroscopy using a 632 nm wavelength excitation (Renishaw Invia Plus laser Raman spectrometer, Renishaw, UK) demonstrated the existence of G band at 1592 cm⁻¹ and D band at 1326 cm⁻¹, respectively. The zeta potential of GO (10 mg/L) in the K-medium was -22.3 ± 2.7 mV.

C. elegans strains and culture. Nematodes used were wild-type N2, mutants of mir-247(n4505), goa-1(sa734), ceh-18(ok1082), pkc-1(ok563), dgk-1(sy428), and pkc-1(ok563);dgk-1(sy428), and transgenic strains of Ex(Punc-14-mir-247), goa-1(sa734)Ex(Punc-14-goa-1), goa-1(sa734)Ex(Pmyo-3-goa-1), goa-1(sa734)
Ex(Pmyo-2-goat-1), Is(Punc-14-goat-1), Is(Punc-14-goat-1); dgk-1(1x428), and Is(Punc-14-goat-1); pck-1(1xok563). Some of the used strains were from Caenorhabditis Genetics Center. Nematodes were maintained on normal nematode growth medium (NGM) plates seeded with Escherichia coli OP50 at 20 °C. The animals were lyed with a bleaching mixture (0.45 M NaOH, 2% HOCI) in order to separate the eggs from the adults, which allow us to obtain the age synchronous L1-larvae populations.

**Exposure and toxicity assessment.** After sonication (40 kHz, 100 W, 30 min), GO was dispersed in the K medium to prepare a stock solution (1 mg/mL), which was diluted with the K medium to obtain the working solution. Prolonged exposure (from L1-larvae to adult day-1) was performed in liquid K medium at 20 °C in the presence of food (OP50).

Head thrash and body bend were used to assess the locomotion behavior. The method was performed under the dissecting microscope by eyes as described previously. Fifty nematodes were examined per treatment.

Intestinal ROS production assay was performed as described previously. After exposure, the nematodes were transferred to 1 μM 5′,6′-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H₂DCFDA). After incubation (3 h in the dark), the nematodes were analyzed under a laser scanning confocal microscope (excitation wavelength, 488 nm; emission filter, 510 nm). Intestinal ROS signals were semi-quantified in comparison to autofluorescence. Sixty nematodes were examined per treatment.

**Reverse-transcription and quantitative real-time polymerase chain reaction (PCR).** Total RNA was isolated from the nematodes using Trizol reagent (Invitrogen, UK) according manufacturer’s protocol. Purity and concentration of RNA were evaluated by a ratio of OD260/280 using a spectrophotometer. The extracted RNA was used for the cDNA synthesis. The relative expression levels of targeted genes were determined by real-time PCR in an ABI 7500 real-time PCR system with EvaGreen (Biotium, USA). All reactions were performed in triplicate. Relative quantification of targeted gene was expressed as the ratio (targeted gene/reference gene tba-1 encoding a tubulin). The related primer information for DNA constructs was shown in Table S1.

**DNA constructs and germline transformation.** To generate entry vector carrying promoter sequence, promoter region for myo-2 gene specially expressed in pharynx, promoter region for myo-3 gene specially expressed in muscle, or promoter region for unc-14 gene specially expressed in neurons was amplified by PCR from wild-type C. elegans genomic DNA. The promoter fragment was inserted into pPD95_77 vector in the sense orientation. goa-1/C26C6.2.1 cDNA containing or lacking 3′-UTR was amplified by PCR, and inserted into corresponding entry vector carrying the myo-2, myo-3, or unc-14 promoter sequence. Transformation was performed by coinjecting testing DNA (10–40 μg/mL) and marker DNA of Pdop-1::rfp (60 μg/mL) into the gonad of nematodes as described. The related primer information for DNA constructs was shown in Table S2.

**RNA interference (RNAi).** RNAi assay was performed basically as described. The nematodes were fed with E. coli strain HT115 (DE3) expressing double-stranded RNA for the examined gene. After grown in LB broth containing ampicillin (100 μg/mL), E. coli HT115 (DE3) expressing double-stranded RNA for the examined gene was plated onto NGM containing ampicillin (100 μg/mL) and isopropyl 1-thio-β-D-galactopyranoside (IPTG, 5 mM). L1 larvae were transferred onto certain RNAi plates until the nematodes became the gravid. The gravid nematode growth medium (NGM) plates seeded with Escherichia coli OP50 at 20 °C52. The animals were lysed after sonication (40 kHz, 100 W, 30 min), GO was dispersed in the K medium to prepare a stock solution (1 mg/mL), which was diluted with the K medium to obtain the working solution. Prolonged exposure (from L1-larvae to adult day-1) was performed in liquid K medium at 20 °C in the presence of food (OP50).

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**Statistical analysis.** Data in this article were expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS 12.0 software (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA), and probability levels of 0.05 and 0.01 were considered statistically significant.

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Author Contributions
D. Wang designed the research. P. Liu, H. Shao, X. Ding, R. Yang and Q. Rui performed the experiments. D. Wang wrote the paper.

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