Inhibition of lipid peroxidation during the reproductive period extends the lifespan of Caenorhabditis elegans

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Glutathione peroxidase 4 (GPx4) is a unique antioxidant enzyme that directly reduces the phospholipid hydroperoxides (PLOOH) generated in biomembranes using glutathione as the reductant. We have previously reported that the Caenorhabditis elegans gpx-quad mutant, which lacks all homologous genes of GPX4 has a reduced lifespan compared with the wild-type. However, the mechanisms underlying the lifespan reduction remain unclear. By monitoring the change in PLOOH production with age, we found that PLOOH was elevated in the gpx-quad mutants compared with the wild-type during the reproductive period. Administration of vitamin E not only reduced the PLOOH content but also prolonged the lifespan of the gpx-quad mutants. In contrast, vitamin C did not extend the lifespan of the gpx-quad mutants. Interestingly, we found that the inhibition of lipid peroxidation by vitamin E during 5 to 10 days after hatching is important to extend the lifespan of C. elegans. These results suggest that production of PLOOH during the reproductive period strongly influences the lifespan of C. elegans.

Key Words: GPX4, phospholipid hydroperoxides, lifespan, vitamin E, C. elegans

In aerobic organisms, highly toxic reactive oxygen species are constantly generated by the incomplete reduction of molecular oxygen, and these are subsequently degraded by antioxidant defense mechanisms. Membrane phospholipids containing polyunsaturated fatty acids (PUFA) are highly susceptible to peroxidation triggered by free radicals, thereby leading to the generation of phospholipid hydroperoxides (PLOOH). The decomposition of PLOOH results in the formation of highly reactive electrophiles such as 4-hydroxynonenal, which covalently modify DNA and proteins. Therefore, the removal of PLOOH is important for the protection of macromolecules from oxidative damage.

The free-living nematode Caenorhabditis elegans (C. elegans) is a model organism that can be used for the study of stress defenses and aging because it is genetically tractable, can be easily manipulated, has a short lifespan, and has thousands of mutant strains are available for study. C. elegans synthesizes a wide range of PUFAs such as linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid, which account for about 30% of its total fatty acid content. Although C. elegans is rich in easily oxidizable PUFAs, the mechanism through which they metabolize lipid hydroperoxides remains unclear. To investigate the significance of removal of PLOOH in C. elegans, we focused on studying the function of glutathione peroxidase 4 (GPX4, also known as phospholipid hydroperoxide glutathione peroxidase PHGPX) in this study. GPX4 is a unique antioxidant enzyme that directly reduces the PLOOH generated in biomembranes, using glutathione as the reductant. We generated quadruple gene deletion mutants (gpx-quad mutants) lacking all C. elegans GPX4 homologs and found that the lifespan of the gpx-quad mutants is significantly shorter than that of the wild-type worms. However, the specific PLOOH which determines the lifespan of C. elegans and at which stage, remains unclear.

Vitamin E is a potent fat-soluble antioxidant that protects membranes against lipid peroxidation by scavenging the reactive peroxy radicals and several reports have shown that the administration of vitamin E prolongs the lifespan of C. elegans. In this study, we monitored the change in PLOOH production with aging in gpx-quad mutants and found that the production of several PLOOH species were significantly enhanced in 10- or 20-day-old gpx-quad mutants as compared with the wild-type. Administration of vitamin E not only suppressed the accumulation of PLOOH but also prolonged the lifespan of gpx-quad mutants. By limiting the period of vitamin E administration and evaluating the life-prolonging effects, we found that the intake of vitamin E during reproductive periods significantly extended the lifespan of C. elegans.

Materials and Methods

General methods and strains. The maintenance and genetic manipulation of C. elegans was conducted as previously described. The Bristol strain N2 was used as the standard wild-type strain. The quadruple mutant strain gpx-1(tm2100); gpx-2(tm2895); gpx-6(tm2525); gpx-7(ksu1) was generated as previously described and each mutant was backcrossed seven times.

Lifespan analysis. One hundred hermaphrodites were placed in nematode growth medium (NGM) plates seeded with Escherichia coli OP50 (10 worms per plate). The worms were incubated at 20°C and transferred to fresh plates every other day until they stopped egg-laying. Hatching was the starting point for the lifespan determination and the organisms which survived were counted daily. If the worms did not respond to a mechanical stimulus, they were scored as dead. Lost, “bagged”, and exploded animals were excluded from the analysis. Survival was plotted by the Kaplan-Meier method and scored for significance with the log-rank test.

Sample preparation for liquid chromatography-mass spectrometry (LC-MS). Phospholipids were isolated by solid phase extraction. About 10,000 hermaphrodites were sonicated with...
1 ml of methanol containing an internal standard (17:0-lysoPC) and extracted after stirring for 1 h at 4°C. After a brief centrifugation (2,000 rpm, 4°C, 5 min), the supernatant was diluted with 10 volumes of water adjusted to pH 3.0 with 0.1 N HCl. The samples were then applied to C18 Sep-Pak Vac RC (500 mg) cartridge (Waters, Milford, MA), which was pre-conditioned with 20 ml of methanol and 20 ml of water. The cartridge was washed with 20 ml of water followed by 10 ml of hexane to remove cholesterol and neutral lipids. The oxidized phospholipids were eluted with 10 ml of methanol. The extracted lipids were dried under a gentle stream of nitrogen, dissolved in 150 μl of methanol and 20 ml of water. The cartridge was washed with 20 ml of methanol and 20 ml of water. The oxidized phospholipids were eluted with 10 ml of methanol. The extracted lipids were dried under a gentle stream of nitrogen, dissolved in 150 μl of methanol, and stored at –80°C until further use.

**LC-ESI-MS/MS analysis.** The LC-ESI-MS/MS analysis was performed using QTRAP 4500 quadrupole linear ion trap hybrid mass spectrometer (AB Sciex, Concord, ON, Canada) coupled to a Nexera XR high-performance liquid chromatography system (Shimadzu Co., Kyoto, Japan). The sample was subjected to LC-ESI-MS/MS analysis using the XBridge BEH C18 column (3.5 μm, 150 mm × 1.0 mm, Waters). Sample (10 μl) was injected by the autosampler and the phospholipid fractions were separated by a step gradient with mobile phase A (acetonitrile:methanol:water = 2:2:1 v/v/v containing 0.1% formic acid and 0.028% ammonia) and mobile phase B (isopropanol containing 0.1% formic acid and 0.028% ammonia) in the following ratios: 100:0 (0–5 min), 50:50 (5–25 min), 50:50 (25–59 min), 100:0 (59–60 min), and 100:0 (60–75 min) at a flow rate of 70 μl/min and a column temperature of 30°C. Multiple reaction monitoring was carried out to detect specific oxidized phospholipids. MS/MS analysis was performed in negative ion mode with the following settings: ion spray voltage: −4,500 V; curtain gas (N2): 30 arbitrary units; collision gas (N2): medium; declustering potential: −60 V; collision energy: −40 V; and temperature: 500°C. For the detection of phosphatidylethanolamine (PE), deprotonated ions ([M-H]+) were selected as the precursor ion and the fatty acyl chains were selected as the product ion ([M-H]+). For the detection of PC, formate adduct ions ([M+HCOO]−) were selected as the precursor ion and the fatty acyl chains were selected as product ion ([M-H-HCOO]+). Administration of vitamins. Embryos were synchronized by hypochlorite bleaching, hatched overnight, and then cultured on NGM plates containing 200 μg/ml of vitamin E (D-α-tocopherol #T3634), Sigma-Aldrich, St. Louis, MO) or 10 mM of vitamin C (Wako chemicals Co. Ltd., Osaka, Japan). 0.1% (v/v) ethanol and 200 μg/ml of Tween-80 were added to NGM plates to dissolve vitamin E.

**Results**

**Peroxidation of phospholipids is elevated in gpx-quad mutants.** In order to investigate the role of PLOOH in the regulation of *C. elegans* lifespan, we used gpx-quad mutants in which the four *gpx* genes (*gpx-1, gpx-2, gpx-6, and gpx-7*) were deleted. These *gpx* genes encode for the *C. elegans* homologs of mammalian GPx4, a unique antioxidant enzyme that directly reduces the phospholipid hydroperoxides in biomembranes. Similar to our previous report, the lifespan of the gpx-quad mutants was significantly shorter than that of the wild-type (Fig. 1A). On the other hand, administration of vitamin E extended the lifespan in wild-type worms (Fig. 1B). These results suggest that the production of PLOOH might have a role in regulating the lifespan of *C. elegans*.

*C. elegans* synthesizes PUFAs such as linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), and eicosapentaenoic acid (20:5), and the phospholipids contain these unsaturated fatty acids. Thus, we determined the change in PLOOH levels produced by the peroxidation of unsaturated fatty acid moieties during the *C. elegans* lifespan (5, 10, and 20 days) using LC-ESI-MS/MS. We first examined the peroxidation of PE, the most abundant phospholipid class in *C. elegans*.[22] In gpx-quad mutants, the levels of several PE hydroperoxide (PEOOH) molecules including PE 18:0/18:2OOH, PE 18:1/18:2OOH, PE e18:0/18:2OOH, PE p18:0/18:2OOH, PE 18:1/20:4OOH, PE e18:0/20:4OOH, and PE 18:1/20:5OOH at 10 days after hatching were significantly higher than those in the wild-type (Fig. 2). We next examined the peroxidation of phosphatidylycholine (PC), another major class of phospholipids in *C. elegans*. We found that the production of several PC hydroperoxides (PCOOH) such as PC 18:0/18:2OOH, PC e18:1/18:2OOH, PC 18:0/18:2OOH, PC e18:0/18:2OOH, PC e18:0/20:4OOH, PC 18:1/20:5OOH at 10 or 20 days after hatching were significantly enhanced in the gpx-quad mutants compared with the wild-type (Fig. 3). Interestingly, in both classes of phospholipids, the production of PLOOH decreased inversely with age and the most significant differences between the wild-type and the gpx-quad mutants were observed in 10- or 20-day-old worms. These results suggest that excessive peroxidation of phospholipids shortens the lifespan of *C. elegans* in the gpx-quad mutants.

**Vitamin E extends lifespan by inhibiting PEOOH and PCOOH production in gpx-quad mutants.** Vitamin E is known to inhibit non-enzymatic lipid peroxidation by terminating the radical chain reaction.[16,17] To examine whether the production of PEOOH and PCOOH shortens the lifespan of *C. elegans* or not,
we administered vitamin E to the \textit{gpx-quad} mutants. Since vitamin E was insoluble in the NGM medium at a concentration higher than 200 \text{mg/ml}, the lifespan of \textit{gpx-quad} mutants was monitored at that concentration of vitamin E. As shown in Fig. 4A, the lifespan of \textit{gpx-quad} mutants was significantly extended by vitamin E, suggesting that the inhibition of PEOOH and PCOOH production extends \textit{C. elegans} lifespan. To confirm this hypothesis, we determined the level of lipid peroxidation in the \textit{gpx-quad} mutants by treatment with vitamin E. PEOOH and PCOOH content was found to be significantly decreased at 5 days after hatching following vitamin E treatment in \textit{gpx-quad} mutants (Fig. 5). In addition, a water-soluble antioxidant (10 mM vitamin C) did not extend the lifespan in \textit{gpx-quad} mutants (Fig. 4B). These data indicate that the production of PLOOH is a key determinant.

Fig. 2. PE hydroperoxide levels are increased in aged \textit{gpx-quad} mutant. Content of PE containing 18:2, 18:3, 20:4, and 20:5-OOH in 5-, 10-, and 20-day-old worms was analyzed by LC-ESI-MS/MS. The data represent the mean ± SD of three independent experiments (*\textit{p}<0.05, Student’s \textit{t} test). The abbreviations used are: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid; and 20:5, eicosapentaenoic acid. Peroxidized fatty acids are indicated with “OOH”. e and p mean ether and plasmenyl linkages, respectively.
of *C. elegans* lifespan which is extended by vitamin E through inhibition of PEOOH and PCOOH production in *gpx*-quad mutants.

**Administration of vitamin E until 10 days after hatching extends *C. elegans* lifespan.** Since the content of several PEOOH and PCOOH species were significantly higher in 10-day-old *gpx*-quad mutants than in the 10-day-old wild-type organisms (Fig. 2 and 3), we hypothesized that inhibition of lipid peroxidation around 10 days after hatching is important for extension of *C. elegans* lifespan. As shown by Fig. 4 and 5, the worms could be administered vitamin E throughout their lifetime. To elucidate the critical period for the life-extension, vitamin E was administered to the worms specifically at several stages of the lifecycle.

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**Fig. 3.** PC hydroperoxide levels are increased in aged *gpx*-quad mutant. Content of PC containing 18:2, 18:3, 20:4, and 20:5-OOH in 5-, 10-, and 20-day old worms was analyzed by LC-ESI-MS/MS. The data represent the mean ± SD of three independent experiments (*p*<0.05, Student’s t test). The abbreviations used are: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid; and 20:5, eicosapentaenoic acid. Peroxidized fatty acids are indicated with “OOH”. e and p mean ether and plasmienyl linkages, respectively.
Fig. 4. Vitamin E extends lifespan in gpx-quad mutants. Survival plots of the gpx-quad mutant treated with or without vitamin E (A) or vitamin C (B) are shown. The percentage of live animals was plotted against the days after hatching. The survival plots represent the average of three independent experiments. The p value of log-tank test is shown in the higher right corner of each figure.

Fig. 5. Vitamin E reduces PE and PC hydroperoxide levels in gpx-quad mutant. Content of PE/PC containing 18:2 and 20:5-OOH in 5-, 10-, and 20-day old worms treated with or without vitamin E was analyzed by LC-ESI-MS/MS. The data represent the mean ± SD of three independent experiments (*p<0.05, Student’s t test). The abbreviations used are: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; and 20:5, eicosapentaenoic acid. Peroxidized fatty acids are indicated with “OOH”. e and p mean ether and plasmenyl linkages, respectively.
and its effects were monitored in the wild-type and \textit{gpx}-quad mutants. At 5 days after hatching, vitamin E extended the lifespan of wild-type animals (Fig. 6A). Since the life-prolonging effects of vitamin E were not observed when administered at 10 days after hatching (Fig. 6C), we further focused on the period from days 5–10 as the critical period of life-extension by vitamin E. As shown in Fig. 6E, we found that vitamin E treatment from 5 to 10 days after hatching could extend the lifespan of wild-type worms. Surprisingly, vitamin E did not extend the lifespan in the \textit{gpx}-quad mutants administered later than 5 days after hatching (Fig. 6B, D, and F). Collectively, these data indicate that inhibition of lipid peroxidation from 5 to 10 days after hatching is required for the extension of \textit{C. elegans} lifespan.

**Discussion**

We have previously reported that the deletion of four \textit{gpx} genes shortens the lifespan of \textit{C. elegans}. However, it remained unclear
which PLOOH and at which stage of the life cycle determined the lifespan of *C. elegans*.\(^{(19)}\) In this study, analysis of the PLOOH content led to the observation that various phospholipids containing PUFA were peroxidized in gpx-quad mutants. In addition, we found that the inhibition of lipid peroxidation by vitamin E from 5 to 10 days after hatching is important for the extension of *C. elegans* lifespan.

A number of reports have shown that the lifespan of *C. elegans* is affected by various antioxidants, such as coenzyme Q10, curcumin, kaempferol, and EUK-8.\(^{(23)}\) In this study, we found that vitamin E, a fat-soluble antioxidant, has life-prolonging effects, but vitamin C, a water-soluble antioxidant, does not have such effects. These data clearly indicate that protection of fat-soluble components from oxidative stress is needed for extending the lifespan of *C. elegans*. Vitamin C regenerates vitamin E by reaction with the vitamin E radicals, thereby maintaining the vitamin E redox cycle.\(^{(24)}\) Since no detectable amount of vitamin E was observed in *C. elegans* (data not shown), life-prolonging effects might not be observed when only vitamin C is administered. In addition, Patananan et al.\(^{(25)}\) have revealed that *C. elegans* synthesizes vitamin C by itself. This may partially explain vitamin C treatment did not extend the lifespan, as the worms have enough amount of vitamin C in the body under the experimental condition. Although this study showed that the inhibition of lipid peroxidation is important in extending the lifespan of *C. elegans*, it is still unclear whether the lifespan was shortened by PLOOH or by its degradation products. A previous report has shown that overexpression of glutathione S-transferase (gst-10) which metabolizes 4-hydroxynonenal, an aldehydic lipid peroxidation product, extends the lifespan of *C. elegans*.\(^{(26)}\) In the gpx-quad mutants, we found a significant increase in PLOOH containing linoleic acid the degradation of which generates 4-hydroxynonenal. Determination of the content of degradation products of lipid hydroperoxides will therefore provide valuable insights into the lifespan regulation of *C. elegans*.

A mature hermaphrodite worm begins laying eggs after three days of hatching and continues egg-laying for a further three to four days. Thus, the period of 5 to 10 days after hatching includes the reproductive period. In *C. elegans*, analogs of vertebrate low-density lipoproteins are synthesized and oxidized in the gut which stimulate germline development.\(^{(27)}\) Even though the mechanism of lipoprotein oxidation remains unclear, it is possible that excessively produced reactive oxygen species attack phospholipids in the gut. Since the gut is the primary tissue in which the worm gpx-genes are expressed,\(^{(15)}\) PLOOH generated during the reproductive period might escape reduction by gpx and therefore shorten the lifespan in the gpx-quad mutants. Administration of vitamin E during the reproductive periods significantly extended the lifespan of the wild-type but not of the gpx-quad mutants. We speculate that the vitamin E content in gpx-quad mutants was not sufficient for extending their lifespan, but unfortunately it is impossible to increase the content of vitamin E because of its insolubility in media.

Since our lipidomic analysis revealed peroxidation of various phospholipid species in *C. elegans*, we infer that phospholipid peroxidation occurs as a non-enzymatic reaction in worms. Since the worms are too small for an anatomical analysis, it is difficult to quantify the content of PLOOH in individual tissues. Identification of the PLOOH-generating tissues by imaging mass spectrometry is necessary and worthy of future investigation.

### Author Contributions

TS and HI conceived the projects and designed the experiments. TS, KM, and YT contributed equally in performing the experiments. TS wrote the manuscript.

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### Abbreviations

- ESI: electrospray ionization
- GPx: glutathione peroxidase
- LC: liquid chromatography
- MS: mass spectrometry
- NGM: nematode growth medium
- PC: phosphatidylcholine
- PCOOH: phosphatidylcholine hydroperoxide
- PE: phosphatidylethanolamine
- PEOOH: phosphatidylethanolamine hydroperoxide
- PLOOH: phospholipid hydroperoxide
- PUFA: polyunsaturated fatty acids

### Conflict of Interest

No potential conflicts of interest were disclosed.

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