Effects of Tocotrienols on Life Span and Protein Carbonylation in Caenorhabditis elegans

Hiroshi A dachi1,2 and Naoaki Ishii2

1Life Science Research Center, Lion Corporation, Kanagawa, Japan.
2Department of Molecular Life Science, Tokai University School of Medicine, Kanagawa, Japan.

To assess the efficiency of tocotrienols against oxidative damage, we have demonstrated in a model-system nematode, Caenorhabditis elegans, that tocotrienol administration reduced the accumulation of protein carbonyl (a good indicator of oxidative damage during aging) and consequently extended the mean life span (LS), but not the maximum LS. Conversely, α-tocopherol acetate did not affect these parameters. As a way to evaluate the protective ability of tocotrienols against oxidative stress, the life spans of animals administrated tocotrienols before or after exposure to ultraviolet B-induced oxidative stress were measured. Ultraviolet B irradiation shortened the mean LS of animals, whereas preadministration of tocotrienols recovered the mean LS to that of unirradiated animals. Interestingly, postadministration also extended the mean LS more than that of unirradiated animals, and administration through the LS conferred greater protection. Thus, the administration of tocotrienols to animals results in a reduction of oxidative stress risks. These data indicated that tocotrienols merit further investigation as possible agents for antiaging and oxidative stress prevention. In addition, they suggest that C. elegans will continue to provide provocative clues into the mechanisms of aging.

There is growing interest in the role of oxygen free radicals in the aging process (1). The free radical theory of aging states that (i) highly reactive, unstable oxyradicals are constantly generated in aerobic organisms (2,3); (ii) the concentration of reaction products by free radicals increases during aging under physiological conditions (4,5); and (iii) oxidative stress such as hyperoxia or ultraviolet (UV) radiation induces the accumulation of oxidative molecular damage (6,7). Insufficiency in both or either scavenging and repair systems against oxidative stress may cause accumulation of oxidative molecular damage, which consequently leads to homeostatic disturbance during aging (8–10).

Many research groups have recently been exploring the possibility that oxidative damage can be prevented. Carney and colleagues demonstrated that the administration of phenylbutyltintrione, a radical trapping agent, prevented the accumulation of oxidized protein in gerbil brains and recovered impaired short-term memory (11). Chronic ultraviolet B (UVB) irradiation induction of visible skin changes, histological alterations, and tumors were reduced with a topical treatment of superoxide-scavenging antioxidants, such as α-tocopherol (α-Toc), ascorbic acid, propyl gallate (12), and conjugated hexadiens (13). These reports indicate that a reduction in the level of intracellular oxidized damage may result in functional improvement.

Vitamin E is the major chain-breaking antioxidant that functions in vivo and is no doubt crucial in preventing the propagation of free radical damage in membranes (14,15). Vitamin E is the collective name for two basic structures: tocopherols and tocotrienols (Figure 1). Tocotrienols differ from tocopherols in that they have an isoprenoid instead of a phytol side chain (16). Many studies have been conducted to understand the role of tocopherols, but not tocotrienols, because of their quantitative differentiation in vivo. The percentage distribution of vitamin E homologues in hairless mouse tissue is more than 90% tocopherols, whereas tocotrienols constitute less than 10% in skin and 1% in other organs (17). Although α-tocotrienol has only one third the biological activity of α-Toc (18,19), it has 40–60 times higher antioxidant potency (20). However, a role of tocotrienols in the aging process has yet to be unequivocally demonstrated.

Much of our understanding of the biology of aging has come from organisms such as mice and rats, but significant progress has been made in the past decade with invertebrates such as the house fly and the nematode Caenorhabditis elegans (21). We previously demonstrated that protein carbonyl, a specific indicator of oxidative damage to proteins (22–26), accumulated during aging in C. elegans. The oxidative stress-sensitive strain (mev-1) accumulated protein carbonyl at a faster rate than did the wild type, whereas oxidative stress-resistant strains (age-1, daf-2, daf-2:daf-12) exhibited no obvious increases (10,27). In addition, protein carbonyl contents were not only inversely correlated with the life expectancy of the nematodes, but they increased under oxidative stress in the form of hyperoxia (10). These results suggest that genetic or environmental factors that reduce oxidative stress may minimize the protein carbonyl accumulation and extend the life span (LS) in C. elegans.

Here we report efforts to determine the relationship between LS and protein carbonyl level as well as the effects of tocotrienols on LS and protein carbonyl accumulation in C. elegans. In addition, as a way to assess the preadministration and postadministration ability of tocotrienols to protect against oxidative stress, the life spans of animals treated with UVB irradiation were determined.

Materials and Methods

Nematode Strains

The wild type (N2) of the nematode C. elegans was from the Caenorhabditis Genetic Center (University of Minne-
sota, St. Paul, MN). Stock maintenance and handling were carried out as described by Brenner (28).

Culture Conditions

Hermaphrodites of the wild type and mutants were grown at 20℃ on nematode growth medium (NG) agar plates, with live bacteria (Escherichia coli OP50, a uracil auxotroph) as food (28). Synchronous cultures were obtained as previously described (29). In brief, eggs were collected from NG agar plates by using sodium hypochlorite (30) and allowed to hatch by incubation overnight at 20℃ in S basal (0.1 M of NaCl and 0.05 M of potassium phosphate buffer, pH 6.0) (28). The newly hatched L1 larvae were cultured on NG agar plates (using 150-mm plastic plates for the protein carbonyl measurement and 35-mm plates for the determination of LS). In order to prevent progeny production, 5-fluoro-2’-deoxyuridine (FudR; Sigma Chemical Co., St. Louis, MO) was added to the NG agar at a final concentration of 40 mM after animals had reached adulthood.

As a way to measure survival and mean LS, the animals were counted every day by elimination of the dead ones. Death was recognized as the loss of spontaneous movement and lack of response to touch with a probe.

Culture in Vitamin E

The extraction of tocotrienol rich fraction (TRF) from palm oil was produced at Lion Corporation (Tokyo, Japan). The final purity of this vitamin E preparation was more than 95%, and its composition was α-tocotrienol, 22%; α-tocopherol, 24%; γ-tocotrienol, 37%; and δ-tocotrienol, 12%; dl-α-Tocopherol acetate was purchased from Sigma Chemical Co. (St. Louis, MO).

The culture with TRF or dl-α-Toc acetate was treated as follows: 80 mg of either antioxidant was dissolved in 1 ml of ethanol containing 80 mg of Tween 80 (Wako Chemical Co., Tokyo), followed by the addition of 9 ml of sterilized distilled water and sonication. A 0.1-ml or 0.01-ml aliquot of the antioxidant solution was added aseptically to 10 ml of autoclaved NG agar medium before solidification. This concentration of 80 or 8 μg of antioxidant per milliliter of medium was used. The control population was grown in a medium containing 0.1% ethanol with Tween 80. It had been established previously that these ingredients did not affect the growth of E. coli and the LS of animals. Each group consisted of 10 plates; the initial animal density was 10–12 animals per plate (n = 10).

Measurement of Protein Carbonyl Content

Animals were collected from the NG agar plates and washed several times with S basal. They were placed on a 40-μm mesh filter in a small chamber filled with S basal, and only living animals that swam out through the mesh within 10 minutes were collected. Live animals were washed several times with S basal, resuspended with 5 mM ethylenediamine tetra-acetic acid (pH8.0), and frozen at −80℃ until use. In each experiment a 10% (vol/vol) homogenate in S basal was made with a Teflon homogenizer. Protein carbonyl content was measured through a slightly modified method of Levine and colleagues (31), which used 2,4-dinitrophenyl hydrazine (DNPH). A 1 ml aliquot of 20% trichloroacetic acid (TCA) was added to 1 ml of the homogenate to precipitate the protein, and the mixture stood 15 minutes at 4℃. After centrifugation at 2,000×g for 15 minutes, the supernatant was discarded, and a 0.5-ml solution of 10-mM DNPH dissolved in 2 M of HCl was added to the protein fraction. In parallel, a blank was prepared by treatment with 2 M of HCl instead of DNPH. After incubation at 20℃ for 60 minutes, 0.5 ml of TCA was added to the sample and stood 15 minutes at 4℃. After centrifugation at 40,000×g for 15 minutes, the supernatant was discarded. The pellet was washed three times with 1 ml of a 1:1 (vol/vol) mixture of 20% TCA with 100% ethanol and ethyl acetate to remove free reagent. The sample was allowed to stand 15 minutes at 4℃, and then it was centrifuged at 40,000×g; the supernatant was discarded at each time. The final precipitated protein was redissolved in 1.5 ml of a 6-M guanidine hydrochloride solution. After incubation for 1 hour at 37℃, the sample was centrifuged at 600×g for 10 minutes. Absorbance at 380 nm was determined, and, with the use of a molar absorption coefficient of 21 mM⁻¹ cm⁻¹, the carbonyl content was calculated as nanomoles of DNPH incorporated (protein carbonyls) per 1 mg of protein. Protein concentration was determined by use of the Pierce bicinechonic acid method (32).

Ultraviolet Light Irradiation

For UV absorption by medium ingredients and antioxidants to be avoided, synchronous animals at the age of 5 days were collected and washed in S-basal and then picked onto an agar plate. Irradiation with UVB was performed with a fluorescent tube (Toshiba, Model FL 20SE, Tokyo, Japan) that emitted mainly at 310 nm for 18 minutes, corresponding to a fluence rate of 26.0 × 10 mJ/cm² as measured with a UV dosimeter (Minolta, Model UM-10, Osaka, Japan), because in preliminary experiments this intensity proved to shorten the life span of the animals. After irradiation, animals were picked up and carefully put onto a new NG agar plate and then maintained at 20℃.

Statistical Analyses

A one-way analysis of variance (ANOVA) was used to analyze experiments for an assessment of increasing protein
carbonyl by age, whereas a two-way ANOVA was used to analyze experiments for an assessment of increasing protein carbonyl by age and administration conditions of TRF. Statistic comparisons of mean LS values among administration conditions of TRF or α-Toc were determined by Duncan’s Multiple Range test.

**Results**

**Life Span and Protein Carbonyl Content**

Figure 2 shows a representative survival curve and protein carbonyl accumulation of animals under physiological conditions. The mean LS was 17.3 days, and protein carbonyl in young animals was 1.1 nmol/mg protein. Afterward, an age-dependent accumulation in protein carbonyl was observed until the end of LS to reach 3.7 nmol/mg at an age of 18 days (p < .01). The curve of protein carbonyl accumulation was a mirror image of the survival curve. These figures are consistent with previous ones, which reported that oxidative damage occurred during the aging process in *C. elegans* (10, 27).

**The Effect of Antioxidant Administration on Life Span and Carbonyl Content**

In order to learn the effects of TRF and α-Toc acetate on oxidative damage under physiological conditions, the mean LS and protein carbonyl contents were determined. Figure 3A shows the survival curves of groups administered different doses of TRF and α-Toc acetate. The administration of TRF to a group extended the mean LS more than that of the control group, 1.5 and 3.2 days for 8 μg/ml (p < .05) and 80 μg/ml (p < .01), respectively, but not the maximum LS. Conversely, there was no difference between the group administered α-Toc acetate and the control group. In contrast, the group administered 80 μg/ml of TRF showed a reduced protein carbonyl accumulation at the age of 15 days (1.9 nmol/mg protein) in comparison with the nonadministered group (2.8 nmol/mg protein; p < .01). However, young animals at the age of 4 days in both groups showed the same level of protein carbonyl (Figure 3B).

**The Protective Efficiency of TRF against UVB-Induced Oxidative Stress**

In order to assess the effect of TRF in protecting against acute oxidative stress, the mean LS of animals exposed to UVB-irradiation-induced oxidative stress were measured (Table 1). The administration of 80 μg/ml just after irradiation until the end of the LS was used, as it was an effective concentration for LS extension under the normal aging process by TRF. As shown in Table 1, UVB irradiation shortened the mean LS by 2.2 days (p < .01), whereas TRF and α-Toc administration recovered the mean LS by 2.5 days (p < .01) and 1.1 days (not significant), respectively.

**Table 1. Mean LS Values of Animals Given TRF and α-Toc Under UVB-Irradiation-Induced Oxidative Stress**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Mean LS ± SD</th>
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<tbody>
<tr>
<td>Non-irradiation</td>
<td>18.5 ± 1.6 ab1</td>
</tr>
<tr>
<td>Irradiation</td>
<td>16.3 ± 1.6 c</td>
</tr>
<tr>
<td>α-Toc</td>
<td>17.4 ± 1.2 ac</td>
</tr>
<tr>
<td>TRF</td>
<td>18.8 ± 1.2 b</td>
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</table>

1Animals were irradiated with UVB (26.0 mJ/cm²) at 5 days of age. TRF and α-Toc were administrated (80 μg/ml) after irradiation to the end of the LS.

2Mean LS values with different letters are significantly different (p < .01) according to Duncan’s Multiple Range test.

3LS, life span; TRF, tocotrienol rich fraction; α-Toc, α-tocopherol; UVB, ultraviolet B. Each group consisted of 10 plates; the initial density per plate was 10–12 animals (n = 10).
For the evaluation of preadministration and postadministration effects, TRF was administered before or after UVB irradiation as follows: 80 μg/ml of TRF was administered (i) for 2 days just before irradiation, (ii) just after irradiation until the end of the LS, and (iii) before and after UV irradiation. As shown in Figure 4, UVB irradiation shortened the mean LS by 2.1 days (p < .01), whereas preadministration recovered the mean LS to that of the nonirradiated group and postadministration extended the mean LS more than the nonirradiated group by 0.8 days (p < .05). It is remarkable that the survival curve of administration before irradiation resembled the survival curve of the nonirradiated group. In addition, administration through the LS to the end showed the most extension of the mean LS.

**DISCUSSION**

The purpose of this study was to evaluate the protective effect of tocotrienols against oxidative damage. The results presented in Figure 2, and our previous results, (10,27) have demonstrated that, in *C. elegans*, (i) oxidative damage in macromolecules accumulated during the aging process, (ii) the quantitative level of oxidative damage in protein was reflected in relative resistance against oxidative stress, and (iii) higher oxidative stress resistance correlated with a longer mean LS. These results strongly suggest that, in *C. elegans*, oxidative stress plays a role in bringing about the changes in cellular function that occur during aging. Moreover, oxidative damage in macromolecules that are induced by free radicals can overwhelm the scavenging and repair systems in a healthy organism.

Extensions of LS following more than 200 μg/ml of α-Toc administration have been reported in the nematodes *Caenorhabditis briggsae* (33), *Turbatrix aceti* (34), and *C. elegans* (35,36). Goldstein and Modric demonstrated that the protection against oxyradical damage afforded by 200 μg/ml of α-Toc in *C. elegans* was due to its ability to scavenge free radicals and stabilize biomembranes (37). In contrast, the effect of tocotrienols in the aging process have yet to be unequivocally demonstrated, despite the fact that they are stronger antioxidants than α-Toc (20, 38, 39). The administration of 8 and 80 μg/ml TRF to *C. elegans* resulted in an extension of the mean LS but not the maximum LS, whereas 80 μg/ml of α-Toc did not extend the mean LS (Figure 3A). These data may reflect the antioxidant activity of TRF. It is supposed that 80 μg/ml of α-Toc is lower than necessary for LS extension, because previous reports showed that the most effective concentration for LS extension was 200 μg/ml (33,36). In contrast, the administration of 80 μg/ml of TRF reduced the level of protein carbonyl (Figure 3B). These results indicate that TRF administration prevented protein oxidation in *C. elegans* under physiological conditions and, consequently, extended the mean LS.

In order to evaluate the efficacy of administered TRF and α-Toc as protection from acute oxidative stress, the life spans of animals irradiated with UVB to induce oxidative stress were measured (Figure 4). UVB-irradiation-induced oxidative stress was used for the following reasons: (i) UVB irradiation generates hydrogen peroxide and hydroxyl radicals in fibroblasts (40) and skin (41), and subsequently causes protein oxidation, lipid peroxidation, and lipophilic antioxidant depletion (41); and (ii) acute UVB-irradiation-induced oxidative stress can be used to evaluate preadministration and postadministration effects (42). The UVB-irradiation-induced damages are supposed to be cumulative cellular alterations, because the somatic tissues of this nematode consist of long-lived postmitotic cells.

TRF administration recovered the mean LS of the UVB irradiation group to that of the nonirradiated group, whereas α-Toc administration did not give a significant recovery. This indicates that TRF has a greater protective function against acute oxidative stress induced by UVB irradiation more than α-Toc does.

For the effects of preadministration or postadministration to be evaluated, TRF was administered before or after UVB irradiation (Figure 4). Irradiation with UVB shortened the mean LS, whereas preadministration of TRF recovered the mean LS to that of the nonirradiated group. Interestingly, postadministration also extended the mean LS to more than that of the nonirradiated group, and administration through the LS to the end seemed additional.

Tocotrienols are also natural membrane antioxidants as α-Toc in many organellas, although in small portions (16). In membranes, tocotrienols show 40–60 times higher antioxidant potency than α-Toc. This occurs because of (i) a higher NADH-, NADPH-, succinate-, and ascorbate-dependent recycling efficiency from chromanoxyl radicals; (ii) a more uniform membrane bilayer distribution; and (iii) a better interaction of chromanols with lipid radicals (20). The patterns of the survival curve in Figure 4 reflect the protective potency of tocotrienols. As Weber and colleagues exhibited in skin (42), tocotrienol administration may provide an efficient way of enriching the animals with different forms of vitamin E that have a potentially higher antioxidative.
tive activity than α-Toc, though more investigations about the distribution of administrated tocotrienols are needed to provide exact reasons.

Postadministration effects seemed to reflect functions not only as chain-breaking antioxidants that prevented the propagation of free radical damage, but also the enhancement of other antioxidant activity, and to the evaluation of other environment-induced oxidative stresses, such as those caused by hyperoxia, paraquat, x-rays, or nitrogen monoxide. Oxidative stress-sensitive mutants in genes that govern the cellular response to oxidative stress [e.g., mev-1 encodes a subunit of the enzyme succinate dehydrogenase cytochrome b, which is a component of complex II of the mitochondrial electron transport chain (46)] should also be useful for the understanding of the mechanism of protection.

In conclusion, we have demonstrated in C. elegans that TRF administration prevented the accumulation of protein carbonyl and consequently extended the mean LS in the physiological condition, whereas α-Toc did not. We found that preadministration or postadministration of TRF had a protective effect against UVB-irradiation-induced oxidative stress. These data indicate that tocotrienols merit further investigation as possible agents for antiaging and oxidative stress-prevention treatment. In addition, C. elegans may hold provocative clues for new ways to investigate the mechanism of aging.

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Address correspondence to Hiroshi Adachi, Life Science Research Center, Lion Corporation, 100 Tajima, Odawara, Kanagawa 256-0811, Japan. E-mail: hadachi@lion.co.jp

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