Dl-α-tocopherol enhances the herbicide 1,1'-dimetyl-4,4'-bipyridium dichloride (paraquat, PQ) genotoxicity in cultured anuran leukocytes

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This cytogenetic and pharmacological study attempts to clarify genotoxicity-enhancement-effect of dl-α-tocopherol (one form of vitamin E) in combination with the herbicide 1,1'-dimetyl-4,4'-bipyridium dichloride (paraquat, PQ) on cultured anuran leukocytes using the superoxide dismutase-mimic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (Mn(III)TMpyP), the hydrogen peroxide-scavenger catalase and the electron donor nicotinamide adenine dinucleotido phosphate (NADPH). PQ only was found to induce structural chromosomal damage in cultured anuran leukocytes in a dose-dependent manner. PQ plus NADPH, which served as positive control, enhanced the genotoxic effect of PQ. Dl-α-tocopherol only did not induce any structural chromosomal damage in the leukocytes. PQ plus dl-α-tocopherol, however, enhanced the genotoxic effect of PQ. PQ plus Mn(III)TMpyP, PQ plus catalase and PQ plus Mn(III)TMpyP plus catalse suppressed the genotoxic effect of PQ. Furthermore, PQ plus dl-α-tocopherol-enhanced chromosomal damage was also inhibited by Mn(III)TMpyP plus catalase. These results suggest that dl-α-tocopherol in combination with PQ functions as an electron donor to PQ.

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Dl-α-tocopherol (α-TH) antioxidant-defense-system functions in inhibiting unsaturated fatty acids (UFAs) autoxidation that is a free radical chain reaction. Functional disruption in this defense system promotes accumulation of reactive oxygen species (ROS) involved in induction of chromosomal damage.

α-TH deficient-induced rabbit liver mitochondria dysfunction causes an increase in liver damage through lipid peroxidation (Tappel and Zalkin 1959), and in addition, α-TH is an essential substance for reproduction of rats (Evans and Bishop 1922; Evans and Burr 1925). These results suggest that α-TH prevents in vivo lipid peroxidation. By this antioxidant action, α-TH inhibits not only *N*-nitrosomorpholine-induced chromosomal damage and DNA lesion in human hepatoma cell line, HepG2 cells (Robichová et al. 2004) but also spontaneous chromosomal damage in lymphocytes derived from Down's syndrome and Fanconi anemia patients (Pincheira et al. 1999, 2001). On the other hand, pro-oxidative α-TH action has been observed in association with human low-density lipoprotein, thus being caused by combination with the redox reaction partner (Bowry et al. 1992; Kontush et al. 1996; Upston et al. 1999). In fact, the most effective vitamin E form d-α-tocopheryl succinate has been shown to enhance the chromosomal damage in human cancer cell lines, Hela cells and ovarian carcinoma cells and to further enhance γ-irradiation-induced chromosomal damage in the same cancer cells, but not to enhance both the damage in human normal fibroblasts (human normal fibroblast cell lines) (Kumar et al. 2002).

Mn(III)TMpyP and CAT are radical scavengers which have specific properties, shown below. Mn(III)TMpyP has dismutation-reaction property that is conversion of superoxide ($\bullet O_2^-$) into hydrogen peroxide (H_2O_2) (Pasternack et al. 1981). This substance has suppressive effect on ischemia/reperfusion-induced $\bullet O_2^-$ generation leading to rat renal DNA damage (Liang et al. 2009). CAT is a crucial endogenous enzyme that catalyzes decomposition of toxic H_2O_2 into water and molecular oxygen (Loew 1900; Kashiwagi 1995; Kashiwagi et al. 1997). Pro-oxidative function of these radical scavengers has not been reported until today.

PQ is worldwide used as herbicide. It causes chromosomal aberrations in cultured Chinese hamster cells (Nicotera et al. 1985; Sofuni and Ishidate 1988; Tanaka and Amano 1989). In the mechanism of PQ cytogenetic toxicity, the PQ cation has been shown to be enzymatically reduced to blue-colored PQ monocation radical by NADPH and NADH (Gage 1968; Dodge and Harris 1970; Bus et al. 1974). PQ monocation radical reacts with molecular oxygen, and then generates $\bullet O_2^-$ that is converted into H_2O_2 by superoxide dismutase (Gage 1968; Bus et al. 1974; Sofuni and Ishidate 1988). Excessive accumulation of H_2O_2 is thought to generate more highly toxic hydroxyl radical by Fenton reaction (Nicotera et al. 1985; Tanaka and Amano 1989), and then to lead to increases in concentration of lipid peroxides, followed by

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chromosomal aberrations (Bus et al. 1976; Nicotera et al. 1985; Tanaka and Amano 1989). PQ-induced oxidative stress has also been shown to cause fatal toxicity and teratogenic toxicity on anuran embryos and tadpoles (Dial and Bauer 1984, Dial and Bauer Dial 1987; Bauer Dial and Dial 1995). The results suggest that anurans have high sensitivity to PQ-induced ROS.

The purpose of the present study is to investigate the possible mechanism of α-TH antioxidant-function-disruption induced by PQ.

MATERIAL AND METHODS

Chemicals

PQ was purchased from Supelco (West Chester, USA). Penicillin-streptomycin solution, phytohemagglutinin M (PHAM), MEM amino acids solution 50 × and MEM vitamin solution 100 × were obtained from Invitrogen Co. Ltd., San Diego, CA, USA. Other chemicals were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA.). α-TH was solubilized in ethanol.

Animal maintenance

Two anuran species, *Rana* (*R.*) *nigromaculata* and *R. ornativentris* were used in the present investigation. Specimens were derived from standard strains maintained in the Institute for Amphibian Biology, Graduate School of Science, Hiroshima Univ. Frogs were treated according to the basic principles expressed in Canadian Council on Animal care, guidelines on: the care and use of wildlife (2003). Specimens of *R. nigromaculata* and *R. ornativentris* were fed on crickets and raised at room temperature (26–27°C) (Kashiwagi et al. 2005).

Culture

Blood samples were collected from frogs anesthetized with diethyl ether and then incubated at 25°C in 1 ml of 70% Hank's balanced salt solution (pH 7.2) containing 630 mg l^{-1} lactose, 24.0 mg l^{-1} L-gultamine, 0.2% (v/v) MEM amino acids solution 50 ×, 0.1% (v/v) MEM vitamin solution 100 ×, 11.8 mg l^{-1} succinate, 5 ~ 10% fetal bovine serum (inactivated at 80°C for 5 min), 2 IU ml^{-1} heparin sodium, 1 µl ml^{-1} PHAM, 100 Units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin for 5 days (Hanada 2002). Each volume of blood per 1 ml of the basal medium was approximately 20 µl.

Chromosome preparation

Chromosome preparations were made according to the method of Hanada (2002). Briefly, after treating with colchicine (final concentration 0.5 µg ml^{-1}) for 4 h, culture mediums were removed. And then blood samples were transferred to each centrifuge tube containing 1 ml of 0.075 M potassium chloride solution and incubated for 20 min at 25°C. After fixation in 200 µl of 1:3 acetic acid/methanol fixative, blood samples were centrifuged at 266 × g for 5 min. One ml of new fixative was poured into centrifuge tubes after supernatant removal. Obtained cell suspensions of blood samples were stored at –20°C until use. Chromosome preparations made using air-drying method were stained with 4% Giemsa solution in sodium phosphate buffer solution at pH 6.8 and observed using light microscopy Axioscope 2 *plus* (ZEISS Co. Ltd.). Photographs of metaphase chromosomes were recorded using the digital camera Nikon D80. One hundred metaphase figures were analyzed for each treatment.

Statistical analysis

Data were analyzed by Chi-square test. *p*-values below 0.05 are considered significant.

RESULTS

Normal R. nigromaculata karyotype and aberrant R. nigromaculata karyotype

Figure 1 shows normal *R. nigromaculata* karyotype and the aberrant karyotype observed in group 7 leukocytes (10^{-6} M PQ + 10^{-5} M α-TH treatment, see 'Result section; *Genotoxic effect of PQ plus α-TH on* R. nigromaculata *leukocytes*' below for further details). Upper figure

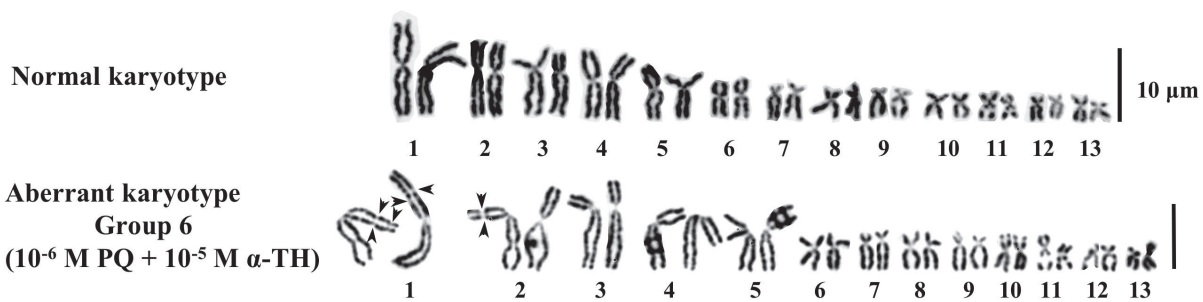

Fig. 1. Normal karyotype of *R. nigromaculata* (upper figure) and aberrant karyotype of *R. nigromaculata* (bottom figure). Arrowheads show breakpoints.

shows the normal karyotype and the bottom figure shows an aberrant karyotype in which the five chromosomal break points on short arms of no.1 chromosomes and the two chromosomal break points on short arm of no. 2 chromosome are examined. In addition, I failed to find the fragile sites of damaged chromosomes no. 1 and no. 2 in group 6 leukocytes, but chromosomal breaks occurred in short arm of no. 1 chromosome in the same group leukocytes are likely to be caused more than the others (data not shown).

Genotoxic effect of PQ on R. ornativentris leukocytes

Figure 2 shows the effect of PQ on cultured leukocytes of *R. ornativentris*. Group-1 leukocytes were treated with 10^{-8} to 10^{-6} M PQ for 6 h. Control leukocytes were incubated in medium to which PQ was not added. A kind of structural chromosomal damage mainly observed in the present investigation was chromosomal break as shown in the bottom figure of Fig. 1. A dose-dependent increase in the frequencies of leukocytes including damaged chromosomes was observed during the PQ treatments. The results show that PQ induces structural chromosomal damage.

Suppressive effect of Mn(III)TMpyP and CAT on PQ-induced structural chromosomal damage in R. ornativentris leukocytes

Figure 3 shows the suppressive effect of Mn(III)TMpyP and CAT on PQ-treated *R. ornativentris* leukocytes. Group 1 leukocytes were incubated in medium containing 10^{-6} M PQ for 6 h, and group 2 leukocytes were incubated

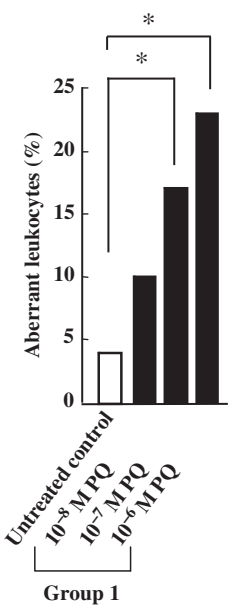

Fig. 2. Increases in the frequency of *R. ornativentris* leukocytes including structurally aberrant chromosome(s) induced by PQ. *Significantly greater than the corresponding value for untreated control leukocytes.

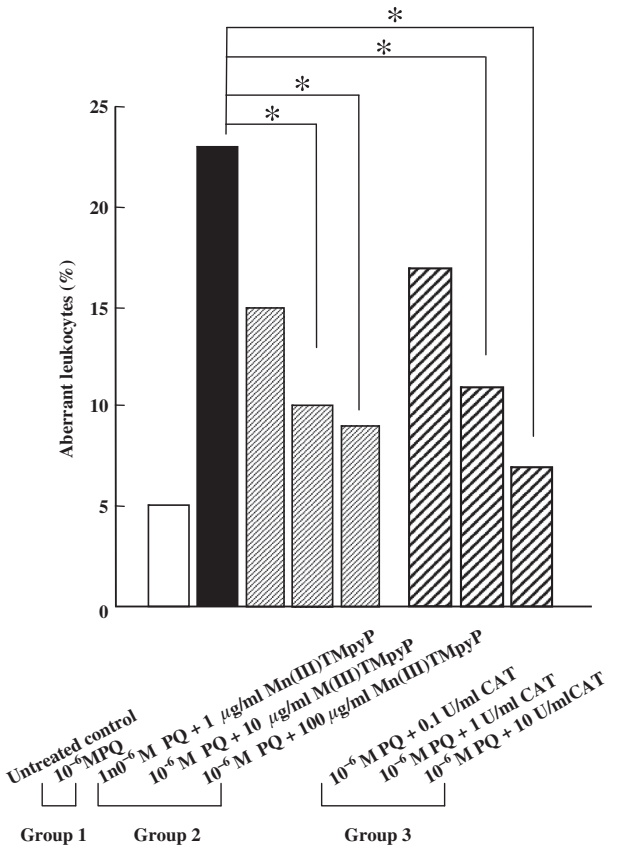

Fig. 3. Effect of PQ plus MnTM(III)pyP and CAT on *R. ornativentris* leukocytes. *Significantly less than the corresponding value for group-1 (10^{-6} M PQ only) leukocytes.

in medium containing 10^{-6} M PQ and 1 to 100 μg ml^{-1} Mn(III)TMpyP for 6 h. Group 3 leukocytes were incubated in medium containing 10^{-6} M PQ and 0.1 to 10 U ml^{-1} CAT for 6 h. Control leukocytes were not exposed to either PQ, Mn(III)TMpyP or CAT. 10^{-6} M PQ only induced the increase in the frequency of leukocytes including structurally aberrant chromosomes by 23%. 10^{-6} M PQ plus 10 to 100 μg ml^{-1} Mn(III)TMpyP suppressed the action of PQ significantly in a dose-dependent manner. 10^{-6} M PQ plus 1 to 10 U ml^{-1} CAT suppressed the action of PQ significantly in a dose-dependent manner. The results show that Mn(III)TMpyP and CAT inhibit induction of structural chromosomal damage by inhibiting superoxide generation and hydrogen peroxide generation.

Suppressive effect of Mn(III)TMpyP plus CAT on PQ-induced structural chromosome damage in R. ornativentris leukocytes

Figure 4 shows the suppressive effect of the combination of Mn(III)TMpyP and CAT on PQ-treated *R. ornativentris* leukocytes. Group 1 leukocytes were incubated in medium containing 10^{-6} M PQ for 6 h. Group 4 leukocytes were

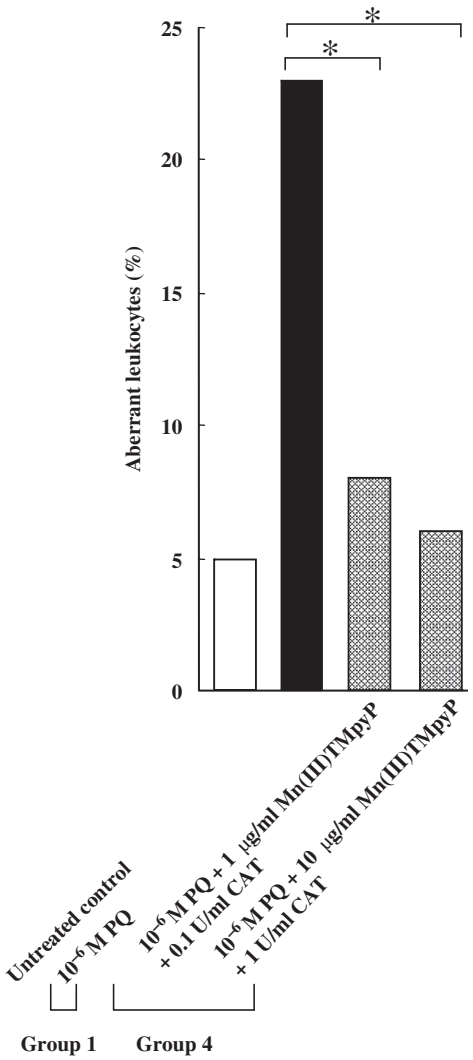

Fig. 4. Effect of PQ plus MnTM(III)pyP plus CAT on *R. nigromaculata* leukocytes. *Significantly less than the corresponding value for group-1 (10^{-6} M PQ only) leukocytes.

incubated in medium containing 10^{-6} M PQ, 1 to 10 μg ml^{-1} Mn(III)TMpyP and 0.1 to 1 U ml^{-1} CAT for 6 h. Control was not exposed to either PQ, Mn(III)TMpyP or CAT. 10^{-6} M PQ only induced the increase in the frequency of leukocytes including structurally aberrant chromosomes by 23%. 10^{-6} M PQ plus 1 to 10 μg ml^{-1} Mn(III) TMpyP plus 0.1 to 1 U ml^{-1} CAT decreased the induction of structural chromosome damage significantly to the control level. The results show that Mn(III)TMpyP plus CAT more strongly inhibits PQ-induced structural chromosomal damage than Mn(III)TMpyP only or CAT only.

Genotoxic effect of PQ plus NADPH on R. nigromaculata leukocytes

Figure 5 shows the enhanced genotoxic effect of PQ plus NADPH on leukocytes of *R. nigromaculata* frogs. Group

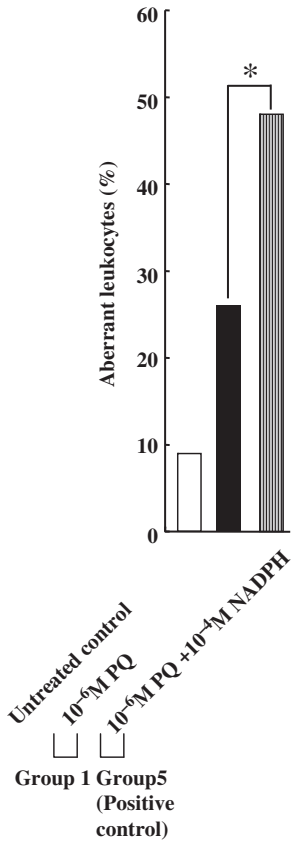

Fig. 5. Effect of PQ plus NADPH on *R. nigromaculata* leukocytes. *Significantly greater than the corresponding value for group-1 (10^{-6} M PQ only) leukocytes.

1 leukocytes were incubated in medium containing 10^{-6} M PQ for 6 h, and group 5 (positive control) leukocytes were incubated in medium containing 10^{-6} M PQ plus 10^{-4} M NADPH for 6 h. Control leukocytes were incubated in medium and not exposed either to NADPH or PQ. 10^{-6} M PQ induced the increase in the frequency of leukocytes including structurally aberrant chromosomes by 26%. 10^{-6} M PQ plus 10^{-4} M NADPH significantly increased the frequency of the aberrant leukocytes by 48%, as expected. The results show that NADPH enhances the genotoxic action of PQ.

Genotoxic effect of α-TH on R. nigromaculata leukocytes

Figure 6 shows no genotoxic effect of α-TH on leukocytes of *R. nigromaculata* frogs. Group 6 leukocytes were incubated in medium containing 10^{-7} to 10^{-5} M α-TH for 6 h. Control leukocytes were incubated in medium and not exposed to α-TH. 10^{-7} to 10^{-5} M α-TH only had no effect on induction of structural chromosomal damage. The results show that α-TH only has no genotoxic effect.

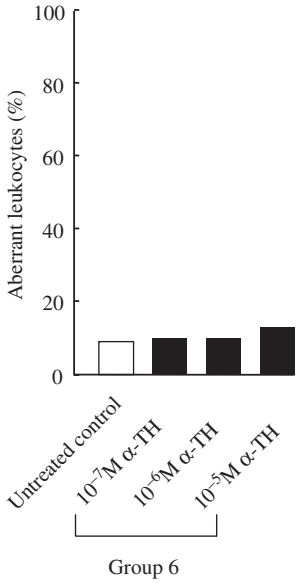

Fig. 6. Genotoxic effect of α-TH on *R. nigromaculata* leukocytes. No significant difference between untreated control leukocytes and α-TH -treated leukocytes.

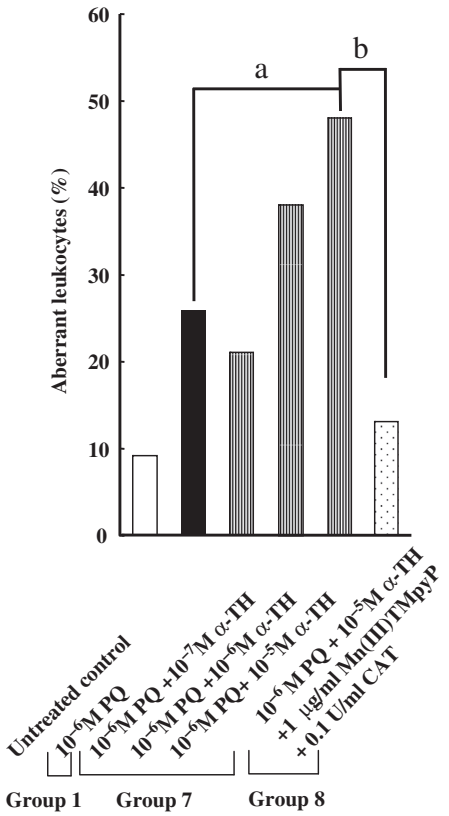

Fig. 7. Effect of PQ plus α-TH on *R. nigromaculata* leukocytes. [a]Significantly greater than the corresponding value for group-1 (10^{-6} M PQ only) leukocytes. [b]Significantly less than the corresponding value for group-7 (10^{-6} M PQ + 10^{-5} M α-TH) leukocytes.

Genotoxic effect of PQ plus α-TH on R. nigromaculata leukocytes

Figure 7 shows the enhanced genotoxic effect of PQ plus α-TH on leukocytes of *R. nigromaculata* frogs. Group1 leukocytes were incubated in medium containing 10^{-6} M PQ for 6 h, and group 7 leukocytes were incubated in medium containing 10^{-6} M PQ and 10^{-7} to 10^{-5} M α-TH for 6 h. Group 8 leukocytes were incubated in medium containing 10^{-6} M PQ, 10^{-5} M α-TH, 1 μg ml^{-1} Mn(III) TMpyP and 0.1 U ml^{-1} CAT for 6 h. Control leukocytes were incubated in medium and not exposed either to Mn(III)TMpyP, CAT, α-TH or PQ. 10^{-6} M PQ induced the increase in the frequency of leukocytes including structurally aberrant chromosomes by 26%. 10^{-6} M PQ plus 10^{-5} M α-TH significantly increased the frequency of the aberrant leukocytes by 48%, although the concentration of α-TH was lower than that of NADPH. The excessive structural chromosomal damage induced by 10^{-6} M PQ plus 10^{-5} M α-TH was significantly inhibited by Mn(III)TMpyP plus CAT. The results show that α-TH enhances the genotoxic action of PQ.

DISCUSSION

Previous studies have shown that PQ enhances ROS generation after enzymatic PQ monocation radical formation in the presence of NADPH, and then induces chromosomal aberrations (Gage 1968; Dodge and Harris 1970; Bus et al. 1974; Nicotera et al. 1985; Tanaka and Amano 1989). In the present study, PQ in combination with NADPH enhanced structural chromosomal damage in cultured anuran leukocytes more than PQ only. The results support the suggestion that NADPH is an electron donor to generate PQ monocation radical. Furthermore, high amount of structural chromosomal damage induced by PQ only was inhibited by MnTM(III)pyP and CAT. The combination of the radical scavengers showed more effective action by the dual inhibitory reaction that Mn(III)TMpyP converted PQ-induced $\bullet O_2^-$ into H_2O_2, and thereafter CAT catalyzed the decomposition of H_2O_2 into water and molecular oxygen. This preventive effect of the radical scavengers on ROS generation shows that PQ-induced oxidative stress results in a high amount of chromosomal damage.

α-TH plays important in vivo roles as an inhibitor of UFAs autoxidation reaction. The typical α-TH antioxidant reaction is hydrogen transfer reaction, as shown below (Mattill 1927; Březina et al. 1990; Frankel 1991):

L-H + various stimulations $\rightarrow$ ($^-H^+$) $\rightarrow$ L• (Initiation of UFAs autoxidation)

$L\bullet + O_2 \rightarrow LOO\bullet$ (lipidic free radical generation)
$LOO\bullet + T\text{–}H \rightarrow LOOH + T\bullet$ (hydrogen transfer reaction by α-TH)
(L-H, UFAs; L•, lipid radical; LOO•, lipid peroxy free radical; LOOH, hydroperoxide; T-H, α-TH; T•, tocopheroxyl radical)

UFAs auto-oxidation is initiated by the various stimulations, irradiation, reaction with metal ion and reaction with other free radicals. The various stimulation-initiated lipid radicals react with molecular oxygen, and then produce unstable lipid peroxy radical. Hydrogen transfer reaction by α-TH converts lipid peroxy radicals into more stable lipid hydroperoxides. Tocopheroxyl radical produced at the α-TH hydrogen transfer reaction is likely to react with another tocopheroxyl radical or lipidic free radicals due to unpaired electron of tocopheroxyl radical (Březina et al. 1990; Frankel 1991). Thus, the α-TH antioxidant system protects human body and animal body against lipid peroxidation. In the present study, α-TH in combination with PQ induced structural chromosomal damage more than NADPH in combination with PQ, in spite of the fact that α-TH only had no cytogenetic toxicity, however. This PQ plus α-TH-enhanced damage was strongly inhibited by the combination with MnTM(III)pyP and CAT. These results suggest that acute increase in level of PQ- radical-formation induced by α-TH occurs in large quantity of ROS generation leading to excessive amount of structural chromosomal damage.

The contradiction of α-TH function as PQ-reducing agent is thought to arise due to extra electron of tocopheroxyl radical derived from α-TH, because α-TH is a chemical agent that has a high potential to react with redox reaction partner.

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