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α-Tocopherol Inhibits the Accumulation of Phospholipid Hydroperoxides in Rat Tissues Induced by 2, 2'-azinobis Hydrochloride*

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The effect of α-tocopherol on the formation and accumulation of phospholipid hydroperoxides, especially of phosphatidylcholine hydroperoxides, in the tissues of 2, 2'-azobis hydrochloride (AAPH) - dosed rats was investigated. In α-tocopherol supplemented rats, the activities of glutathione peroxidase, catalase and superoxide dismutase were significantly inhibited, compared with the AAPH group. AAPH treatment led to oxidation of phospholipids in the liver, lungs, brain, plasma and red blood cells (RBC), resulting in a notable increase in phosphatidylcholine hydroperoxide (PCOOH). All tissues of the rats given an α-tocopherol supplement showed an attenuation of the stimulating effect of AAPH, leading to low levels of formation of PCOOH. Also, the rats injected with AAPH and α-tocopherol showed relatively normal-appearing hepatocytes, except for a little loss of the granules. With regards to the morphological appearance of the liver, it was observed that oral intakes of α-tocopherol resulted in an antioxidant defense against attacks of peroxyl radicals. Thus, we suggest that α-tocopherol is potentially helpful in protecting membrane phospholipids against oxidative damage in vivo.

Key word : α-tocopherol, phosphatidylcholine hydroperoxide, superoxide dismutase, glutathion peroxidase, catalase

INTRODUCTION

Lipid peroxidation has received much attention in connection with its pathological effects and possible contribution to some diseases.[1] Oxidative damage of tissue has also been proposed to be involved in many degenerative diseases and in aging by some investigators.[2] Many studies showed evidence that reactive oxygen species (ROS) play a major role in these pathophysiological events.[3] The effects of antioxidants were examined by comparing the severity of hepatocellular injuries in mice receiving 2, 2'-azobis (2-amidinopropane) hydrochloride (AAPH) plus antioxidant, with those receiving only AAPH.[4] As a result of such experiments, α-tocopherol was proved effective in suppressing hemolysis in the erythrocyte membrane.[5] It was also shown that water-soluble chain-breaking antioxidants, such as ascorbic acid, uric acid, and water-soluble chromanol, suppressed hemolysis in a dose-despondent manner. However, the effects of ROS in biological systems are still not clear because of the difficulty of measuring the generation of oxygen free radicals.

Many assay techniques have been used to measure lipid hydroperoxides in tissues, including the absorbance of conjugated dienes and activity of oxidation using thiobarbituric acid (TBA). However, these measurements are not specific for lipid hydroperoxides. The research therefore requires specific, sensitive, and reproducible procedures to quantify lipid hydroperoxides, and the assay should eliminate interference from other materials. Phospholipids such as phosphatidylcholine (PC) are very important functional components of biological membranes and are also major structural constituents in tissue lipids. Therefore, the measurement of PC is needed to determine the degree of peroxidation of biomembranes and tissue lipids. Miyazawa et al.[6] have developed a chemiluminescence detection-high performance liquid chromatography (CL-HPLC) system for the assay of lipid hydroperoxides, and they expanded this technique to measure PCOOH in human blood plasma and other biological

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materials.[7] We have also used the CL-HPLC system to measure levels of lipid hydroperoxides.[8),9),10)]

Administration of the free radical initiator, AAPH, has been shown to lead to the oxidation of many biomolecules[1)]; however, the degree of oxidative damage differs according to the dose and the route of administration. Therefore, the selection of a suitable free radical initiator is very important, especially in in vivo experiments. The use of AAPH has several merits, one of which is to form peroxyl radicals in aqueous solution without any specific target organs.[12),13)] In the present study, we demonstrated that AAPH (50mg/kg body weight) injections produce hepatoxicity and mortality in the male rat.

AAPH generates carbon radicals by thermal unimolecular decomposition and its carbon radicals react very rapidly with oxygen to give peroxyl radicals.[12)] The intraperitoneally injected AAPH is incorporated into the blood stream and within a few minutes is circulated around the whole body. Among antioxidants, α-tocopherol is well known as a chain-breaking antioxidant. α-tocopherol donates one hydrogen atom to a free radical, converting it to a nonradical product.

It seems likely that damage to biological defense systems results in an increase in lipid peroxidation and a decrease in α-tocopherol. The aim of our current study was to examine whether α-tocopherol treatment inhibit the accumulation of phospholipid hydroperoxides in rats, experimentally induced by AAPH.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (300-350g) were raised on a standard laboratory diet. The animals were randomly divided into three groups; the control animals were injected only with saline; the AAPH animals were injected intraperitoneally with AAPH (50mg/kg body weight, in saline, Wako Co., Japan); and the AAPH+α-tocopherol animals were dosed through a stomach tube with 25mg/kg/day of α-tocopherol diluted with corn oil (Sigma Co.), for 1 week prior to an AAPH injection. Eighteen hours after the AAPH treatment (or after the saline treatment in the case of the control animals), heparinized blood was withdrawn by heart puncture under light diethyl ether anesthesia. The liver was perfused with ice-cold 0.15M saline, and then the brain, liver and lungs were removed.

Lipid extraction

Total lipids were extracted with a mixture of chloroform and methanol (2:1, v/v)[7)] from the liver, brain and lungs. Then 2ml of 0.15M NaCl containing 0.002% butyrate hydroxytoluene (BHT) as antioxidant was added to 500mg of each tissue, and the mixture was homogenized in a teflon-glass homogenizer set in an ice bath. The homogenate obtained was added to 5ml of chloroform/methanol (2:1, v/v) and was mixed vigorously for 1 minute. The mixture was then centrifuged at 2,000 x g for 10 minutes. The lower layer (Chloroform layer) was collected and concentrated in a rotary evaporator, and then dried under a nitrogen stream. The total lipids obtained were diluted with an appropriate amount of chloroform/methanol (2:1. v/v) and 20 μg portions were subjected to the CL-HPLC system[7)].

Determination of phospholipid hydroperoxide (PCOOH)

Lipid peroxidation was assessed by measuring PC-OOH using a Gilson HPLC equipped with a chemiluminescence detector (CLD-110, Tohoku Electric Company, Japan). The Sil-NH2 column (4.6 x 250mm) (Japan Spectroscopic Company, Japan) was eluted with a hexane/isopropanol/methanol/water (5:7:2:1. v/v/v/v) solvent mixture at a speed of 1.0ml/min. Also, a mixture of 10 μg/ml cytochrome c and 2 μg/ml luminol in a 50mM borate buffer (pH 10.0, flow rate 1ml/min) was used as a post-column hydroperoxide-specific luminescent reagent[8),9),10)].

Biochemical Assays

The levels of asparate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as the indices of liver function by using kit reagents (AM 101-K) obtained from A-san Pharmaceutical Co. (Seoul, Korea). The α-tocopherol concentrations in tissues and blood were measured by a fluorescence-HPLC method [14)]. The level of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined by the methods of McCord[15)] and Tappel[16)], respectively. Catalase activity was determined by the method of Aebi.[17)]

Electron micrograph of the liver

For the morphological study, the samples were fixed with modified Dalton's fixative and dehydrated in a graded series of ethanol solutions and embedded in epoxy resin. Ultra-thin sections were prepared on Reihert ultramicrotome, picked up on copper grids, and stained with methanolic uranyl acetate and lead citrate. The specimens were observed with a JM 1200 EX II electron microscope.

Data Analysis

The data were expressed as mean values and standard deviations. Differences among the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range test (SPSS 7.5. SPSS Institute. USA). Differences of $p < 0.05$ were considered significant[18)].

RESULTS

AAPH-induced changes in biochemical parameters

The plasma concentrations of α-tocopherol in the AAPH-administered groups were not significantly different from the control. The levels of plasma AST and ALT in rats dosed with AAPH increased by 2 and 4 times, respectively, compared with the control group. Increases in ALT and AST levels induced by AAPH treatment were significantly inhibited in those animals that were also given an α-tocopherol supplement (Table 1).

Table 1. α-Tocopherol concentration and biochemical parameters in the plasma of rats in three experimental groups.

Group	α-Tocopherol (μg/ml plasma)	Plasma (1 U/L)	
	Plasma	AST	ALT
Con	25.2 ± 5.2	150.0 ± 41.0[a]	33.0 ± 1.4[a]
Con + AAPH	29.5 ± 3.6	295.0 ± 38.1[b]	127.0 ± 36.4[b]
AAPH + α-Toc	29.0 ± 1.3	190.0 ± 32.5[a]	49.5 ± 10.6[a]

Each value represents the mean ± S.D (n=10). [a-b] Mean values with different superscripts are significantly different ($p<0.05$). AST: Aspartate aminotransferase. ALT: Alanine aminotransferase. Con: the rats injected with saline. Con + AAPH: the rats injected with AAPH only. AAPH + α-Toc: The rats injected with AAPH after α-tocopherol supplementation.

Levels of PCOOH in the tissues and blood plasma

Fig 1 shows the concentrations of phosphatidylcholine hydroperoxide (PCOOH) measured by CL-HPLC in tissues and in blood treated or untreated with AAPH. A single peak was obtained by the CL-HPLC system at a retention time of 7 minutes, and this was identified as PCOOH because it was equivalent to the retention time observed with standard PCOOH samples. Each calibration line shows a good proportional correlation between the chemiluminescence peak area calculated by the integrator and the hydroperoxide concentration (data not shown). For the rats in the control group, the amounts of PCOOH present were 490±56 pmol/g in the liver, 413±12 pmol/g in the lung, and 420±21 pmol/g in the brain. For the rats injected with AAPH only, the amounts of PCOOH present were 793±49 pmol/g in the liver, 557±16 pmol/g in the lung, and 816±42 pmol/g in the brain. For the rats injected with AAPH after α-tocopherol supplements, the amounts of PCOOH present were 507±38 pmol/g in the liver, 420±21 pmol/g in the lung, and 535±21 pmol/g in the brain. Levels of PCOOH in the plasma of the control group were below the detection limit, whereas levels of the AAPH group averaged 57.5 pmol/g. The levels of PCOOH of the AAPH plus α-tocopherol group were slightly different to the AAPH group in plasma. Levels of PCOOH in the red blood cells (RBC) of the AAPH group were 2 times higher than in the control group. The results show that all tissues of the α-tocopherol supplemented group achieved low levels of PCOOH formation compared to the AAPH group. Overall, the values obtained suggest that the α-tocopherol supplement inhibits from 50% to 90% of the PCOOH formation, which would result from the AAPH injections.

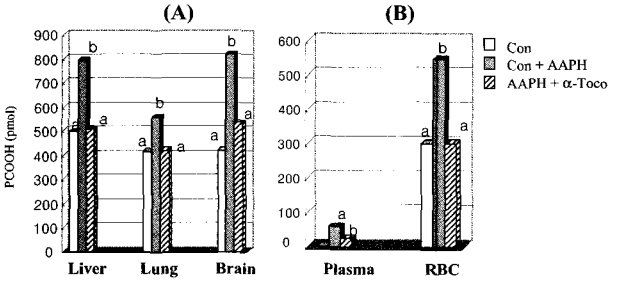

fig 1. Phosphatidylcholine hydroperoxide levels in plasma, RBC, and tissues of rats in three experimental groups

Values are mean±SD (n=10). Con: The rats injected with saline. Con + AAPH: the rats injected with AAPH only. AAPH + α-Toco: the rats injected with AAPH after α-tocopherol supplementation. ND: not determined (below 10 pmol/ml plasma)

The activities of antioxidant enzymes in red blood cells

Table 2 summarizes the activities of glutathione peroxidase (GSH-Px), catalase and superoxide dismutase (SOD) in the three experimental groups. For the AAPH group, the activities of all these enzymes were increased by a factor of 2 (GSH-Px) to 4.1 (SOD) compared with the control. By contrast, the levels of activity of these enzymes in α-tocopherol supplemented group were significantly inhibited (1.1 to 5 times. The results indicated that α-tocopherol was effectively scavenging the active oxygen generated by AAPH.

Table 2. Activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase in the red blood cells of rats in three experimental groups

Antioxidant enzyme/ Groups	GSH-Px (μmol/min/mg protein)	SOD (Units/mg protein)	Catalase (Units/mg protein)
Con	0.18 ± 0.01[a]	2.07 ± 1.90[a]	51.5 ± 9.80[a]
Con + AAPH	0.38 ± 0.10[b]	8.56 ± 1.81[b]	81.4 ± 5.98[b]
AAPH + α-Toc	0.20 ± 0.06[a]	3.09 ± 1.47[a]	60.1 ± 4.9[a]

Each value represents the mean ± S.D (n=10). [a-b] Mean values with different superscripts are significantly different ($p<0.05$). Con: the rats injected with saline. Con + AAPH: the rats injected with AAPH only. AAPH + α-Toc: the rats injected with AAPH after α-tocopherol supplementation.

Electron microscopy

Electron microscopy of a portion of the liver of the control rats revealed hepatocytes that were intact and well preserved (Fig 2-A). The nucleus (N) is seen in the slide portion. The mitochondria are moderately dense

and contain well-formed cristae, endoplasmic reticulae (ER), and granules (G). The AAPH-treated livers resulted in various morphological changes (Fig 2-B). Most of the hepatocytes possessed somewhat swollen mitochondria, which had irregular forms. The nucleus was found to be destroyed in part, and also showed a loss of granules compared with control rats and α-tocopherol- administered rats. By contrast, the rats injected with AAPH after α-tocopherol treatment showed relatively normal-appearing hepatocytes, except that there was a little loss of granules (Fig 2-C).

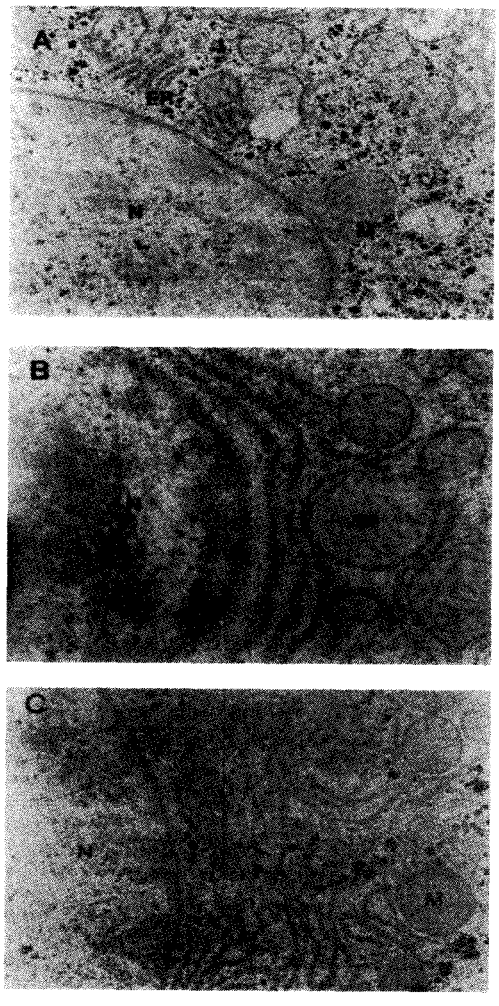

Fig 2. *Electron microscopy of portions of livers from rats in three experimental groups.*

A : Electron microscopy of a portion of a liver from a rat belonging to the control group revealed that the liver was intact and well preserved. The nucleus (N) is seen in the side portion. The mitochondria are moderately dense and contain well-formed cristae, endoplasmic reticulum (ER), and granules (G). Magnification X 5,000.

B : The livers from AAPH-treated rats showed various morphological changes. Most of the hepatocytes manifested somewhat swollen mitochondria with irregular forms. The nucleus was found destroyed in part and has a loss of granules, compared with the control and α -tocopherol administered rats. Magnification X 5,000.

C : The rats treated with AAPH after α-tocopherol supplementation showed relatively normal-appearing hepatocytes, except that there was some loss of granules. Magnification X 5,000.

DISCUSSION

Fat-soluble vitamin E, α-tocopherol, is known to be the principal antioxidant in lipophilic biological systems and is thought to play an important role in protecting biomembranes from radical-induced injury.[19] However, the mechanism responsible for the initiation of biomembrane oxidation and the mechanisms by which α-tocopherol inhibit oxidation still require detailed study.

Lipid peroxidation in biological membranes has received much attention in connection with pathological effects and possible contributions to cancer and aging.[2),20),21)] Aerobic organisms protect their biological membranes from oxygen toxicity by an array of defense systems. Antioxidative molecules can deactivate active species (and their possible precursors) to free radicals, and directly scavenge free radicals, thereby suppressing the generation of free radicals by molecular oxygen.[5] In the case of rats injected with AAPH (50mg/kg body wt.), the levels of PCOOH in plasma, RBC, liver, lung and brain tissues were increased by approximately a factor of 2 (Fig 1). On the other hand, rats given an α-tocopherol supplement were found to have PCOOH levels similar to those of the control group. These results imply that α-tocopherol is closely involved in lowering levels of PCOOH in tissues.

Oral supplementation of α-tocopherol helped to reduce AAPH-induced hepatotoxicity. Plasma AST and ALT levels were 2 to 4 fold higher in rats receiving AAPH injections compared with the control. There were no significant differences in plasma AST and ALT levels between the control and the α-tocopherol treated rats. At 18 hours after the AAPH injection, increases in PCOOH were evident in the tissues and in the blood; this may be related to the fatty infiltration and necrosis in rat tissues induced by AAPH intoxication. Despite the stimulating effects of AAPH, all tissues from rats given an α-tocopherol supplement were found to have low levels of PCOOH. The morphological appearance of the livers of α-tocopherol treated rats was clearly normal; there was no evidence of hepatocellular injury, with no detection of disrupted membranes, swollen configurations of mitochondria, or loss of granules (Fig 2). These results present direct evidence that oral intake of α-tocopherol can act as an antioxidant against free radical damage in tissues.

Enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, also affect biological membranes.[5] For this study, there was no real assay system available, which could be used for the study of various free radical reactions in biological systems. In the control and AAPH plus α-tocopherol groups, both catalase and SOD activities were significantly lower than in the AAPH group. The results suggest that α-tocopherol

significantly inhibited oxidative enzyme activity.

In AAPH initiated lipid peroxidation of tissue, peroxyl radicals are generated in the aqueous phase and then attack membranous phospholipids at their surfaces.[22] Our result indicates that α-tocopherol, related to the chain-breaking phenolic antioxidants, inhibits phospholipid peroxidation by AAPH. Further studies on the physicochemical properties of α-tocopherol are necessary to fully understand their antioxidant activity in membranous phospholipids.

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