Effect of Deficiency of Vitamins C and/or E on Lipoprotein Metabolism in Osteogenic Disorder Shionogi Rat, a Strain Unable to Synthesize Ascorbic Acid

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Summary The effects of vitamin C and/or E deficiency on lipoprotein metabolism were investigated in the inherently scorbutic Osteogenic Disorder Shionogi (ODS) rat. In the vitamin C-deficient (C-def) group, marked increases in plasma VLDL and LDL cholesterol were observed (by comparison with the vitamins C- and E-sufficient control group). In rats kept deficient in both vitamin C and vitamin E (C,E-def), LDL cholesterol was significantly higher than in the C-def group even though the levels of VLDL and HDL cholesterol were similar between the two groups. TBARS values for the LDL fraction in the C-def group were of the same magnitude as in the E-def group, and these values were significantly higher than those obtained for the control group. In the C,E-def group, the values were even higher than in the E-def and C-def groups. The non-denatured PAGE of the LDL fraction indicated the appearance of HDLG in the C-def and C,E-def groups. The SDS-PAGE of the LDL fraction showed increased apo B-48 in the C-def and C,E-def groups and increased apo E in the C,E-def group. Decreased plasma LCAT activity in the E-def, C-def, and C,E-def groups indicated an alteration in HDL metabolism as a result of oxidation. These results suggest that lipid peroxidation and some distinct features of lipoprotein metabolism resulting from vitamin C deficiency become more significant when vitamin E is also deficient along with vitamin C deficiency.

Key Words vitamin C, vitamin E, deficiency, Osteogenic Disorder Shionogi rat, lipoprotein metabolism

Vitamins C and E play significant roles in lipid metabolism. Vitamin C, a well-known major water-soluble antioxidant, traps peroxy radicals in the aqueous phase before they can diffuse into plasma lipids (1, 2). In contrast, vitamin E is a major lipid-soluble chain-breaking antioxidant and strongly inhibits the propagation of lipid peroxidation (3, 4). It has been reported that these two vitamins may act in collaboration in the protection of low-density lipoprotein (LDL) and plasma lipids against free radical-mediated oxidation (5). It has also been suggested that they may act synergistically to suppress lipid oxidation, vitamin C chemically reducing the vitamin E radical and thus regenerating vitamin E (6).

Vitamin C also has a significant effect on plasma cholesterol metabolism (7), as consistently found in animal studies. In guinea pigs, a marginal vitamin C deficiency increases cholesterol levels in the plasma and in a variety of tissues (8–10). Moreover, in vitro studies suggest that the resistance of LDL to oxidation is related to its vitamin E content (11, 12). It has also been demonstrated that in vitro, physiological levels of vitamin C effectively inhibit the oxidative modification of LDL (13). Several studies have suggested that these vitamins may, by acting as antioxidants, protect against lipid peroxidation in vivo. A chronic depletion of plasma antioxidants may generate conditions in the plasma favorable to oxidation (14). In a study of vitamin E feeding in experimental animals fed a diet enriched with cholesterol, pro- and antiatherogenic effects were both noted (15). Further, high-dose vitamin C supplementation reduced oxidative stress in the streptozotocin-induced diabetic rat (16). It is interesting that Igarashi et al. reported the possibility of a synergistic action of vitamins C and E in vivo in both guinea pig and rat (17).

Since rats synthesize vitamin C themselves, guinea pigs were traditionally used for experiments with vitamin C. Some 20 y ago, a mutant rat unable to synthesize vitamin C [the Osteogenic Disorder Shionogi (ODS) rat] was established by Makino and Katagiri, and this rat has been used in studies of the role of vitamin C in lipid metabolism (18). In studies in which ODS rats were fed cholesterol, a vitamin C deficiency in the diet resulted in an accumulation of cholesterol in the plasma and liver, reduced cholesterol 7-α-hydroxylase activity, and reduced the excretion of fecal bile acids [by comparison either with mutant rats fed normal amounts of the vitamin (19, 20) or with normal rats (21)], but the same vitamin deficiency had only a minor effect on cholesterol metabolism in these rats when they...
were fed a normal diet.

The present study was designed to investigate the effect of the deficiencies of vitamins C and/or E on lipoprotein metabolism produced by creating a vitamin E deficiency, besides the vitamin C deficiency inherent in ODS rats.

**MATERIALS AND METHODS**

**Materials.** Cholesterol (99+%), crystallized lyophilized bovine serum albumin, and 1-phosphatidylcholine (egg yolk lecithin; Type III-E) were all obtained from Sigma Chemicals (St. Louis, MO, USA). TLC plates (silica gel 60 plastic sheets) were purchased from Merck (Darmstadt, Germany). [7(N)-3H]-Cholesterol (specific activity, 263 GBq/mmol) was purchased from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK). All other chemicals were of reagent grade or better and were purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Animals and diets.** Male ODS/Shi Jcl-od/od rats were obtained at 6 wk of age from CLEA Japan Inc. (Tokyo, Japan). For 30 d, the rats were fed a basal vitamin C and vitamin E deficient (C, E-def) diet ad libitum. This diet contained 6% vitamin E-stripped corn oil and 25% vitamin-free casein. They were allowed free access to distilled water containing 40 mg ascorbic acid per 100 mL for the same period. This level of ascorbic acid in the drinking water was estimated to be vitamin C sufficient on the basis of the findings of Horio et al. (22). The composition of the basal C, E-def diet is shown in Table 1. Food and drink were provided fresh daily or every second day. The 20 rats fed the basal diet were divided into four equal groups at the end of the 30-d period: (i) a vitamin E and C sufficient (control) (E, C-suf) group that was fed the basal diet plus 20 mg DL-α-tocopherol and had free access to distilled water containing 40 mg ascorbic acid per 100 mL, (ii) a vitamin E deficient and vitamin C sufficient (E-def) group, (iii) a vitamin E-sufficient (E-suf) and vitamin C-deficient (C-def) group, and (iv) a C, E-def group. The rats, which were maintained by the use of pair-feeding with these diets, were kept individually in stainless steel cages at 23°C and 50±5% humidity for a further 30 d. After this, all rats were fasted for 12 h, and blood was then collected from the abdominal aorta under diethyl ether anesthesia; the plasma was used for the following types of analysis.

**Lipoprotein fractionation.** We fractionated plasma lipoproteins by sequential ultracentrifugation, using a Beckman TL-100 at 4°C in a manner similar to that described previously (23). The density ranges for chylomicrons plus very-low density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) was d<1.006 (g/mL), 1.006<d<1.063, and 1.063<d<1.21, respectively, determined with the use of a KBr solution. The isolated lipoprotein fractions were dialyzed against 0.02 M sodium phosphate-buffered saline, 0.15 M NaCl, pH 7.4, and used immediately or stored under N2 at 4°C until use.

**Electrophoresis.** Nondenatured polyacrylamide gel electrophoresis (PAGE) was performed as described by Narayan et al. (24). Samples prestained with 0.5% Sudan black B in propylene glycol were applied to 3.75% Tris-HCl buffered polyacrylamide gel with 2.5% stacking gel, and run at 20 mA constant current in Tris-glycine buffer, pH 8.3.

Sodium dodecyl sulfate (SDS)-PAGE was performed as described by Laemmli (25). It was performed in 12.5% polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) containing 0.1% SDS. One sample was applied per lane after heating in a sample buffer (125 mM Tris HCl, pH 6.8. 4% SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue, and 20% glycerol) at 100°C for 5 min, and run at 20 mA constant current in Tris-glycine buffer containing 0.1% SDS, pH 8.3. Proteins in the gel were stained with Coomassie Brilliant Blue R. The molecular markers used were from a low molecular weight calibration kit purchased from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK).

**Table 1. Composition of the basal experimental diet.**

| (A) Ingredients (g/100 g diet) | (B) Salt mixture (mg/100 g diet) | (C) Vitamin mixture (mg/100 g diet) |
|-------------------------------|-------------------------------|-----------------------------------|
| Vitamin-free casein 25        | K 692                         | Vitamin A 1000 IU                 |
| Corn starch 38                | P 597                         | D3 200 IU                         |
| α-Starch of wheat 10          | Ca 411                        | B3 2.4                            |
| Corn oil stripped of vitamin E 6 | Na 270                       | B2 8.0                            |
| Salt mixture* 6               | Mg 86                         | Be 1.6                            |
| Vitamin mixture** 2           | Fe 41                         | B12 0.001                         |
| Powdered filter paper 8       | Zn 0.4                        | K1 10.4                           |
| Sucrose 5                     | Mn 1.3                        | Biotin 0.04                        |
|                               | Cu 0.08                       | Folic acid 0.4                     |
|                               | I 7.7                         | Ca-pantothenate 10.0               |
|                               |                               | p-aminobenzoic acid 10.0           |
|                               |                               | Niacin 12.0                        |
|                               |                               | Insol 12.0                         |
|                               |                               | Choline-Cl 400.0                   |

* Components of salt mixture (Oriental mixture; Oriental Yeast Co. Ltd., Osaka, Japan) are shown in (B).

** Components of vitamin mixture (vitamin C and E deficient oriental mixture) are shown in (C).
Enzyme assay. The activity of the enzyme lecithin-cholesterol acyltransferase (LCAT) was measured by the use of one of two methods. The first, Stokke and Norum’s method (26), involved the addition of 30 μL of [7N-3H]-cholesterol (333 Bq) in 25 mg/mL bovine serum albumin emulsion to 100 μL of whole human plasma; this was incubated for 4 h at 37°C under N2 with 20 μL of 10.4 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which is an LCAT inhibitor. The reaction was started by the addition of 20 μL of 100 mM 2-mercaptoethanol. Following incubation for 60 min, the lipids were extracted and separated by TLC, using n-hexane-diethyl ether-acetic acid (70 : 30 : 1, v/v). The radioactivity in the cholesterol and cholesterol-ester fractions was determined with a liquid scintillation counter.

In the second method, the enzyme activity was measured in a manner similar to that described previously (27). Lecithin-cholesterol vesicles prepared by the method of Batzri and Korn (28) were used as substrate for the enzyme assay. A typical preparation contained 900 nmol egg yolk phosphatidylcholine and 150 nmol of [7(N)-3H]-cholesterol per mL. The assay mixture consisted of 100 μL of vesicle solution, 15 μg of human apolipoprotein (apo) A-I, 4 μM 2-mercaptoethanol, 0.7 mM EDTA, 2.5 mg of bovine serum albumin, and 3 μL plasma as an enzyme source in a final volume of 250 μL of 39.2 mM sodium phosphate buffer, pH 7.4. Test tubes containing assay mixture were reacted for 60 min at 37°C under N2. The rest of the procedure was the same as in the first method.

Assays. Plasma ascorbic acid was quantified by the o-phenylenediamine fluorescence method (29).

The protein content in each sample was determined by the method of Lowry et al. (30), with BSA as a standard. The total cholesterol and free cholesterol, triglyceride, and phospholipid contents of the sample were determined enzymatically with kits from Wako Pure Chemical Industries. Lipid peroxides were measured as thiobarbituric acid-reactive substances (TBARS) according to Yagi’s method (31), with 1,1,3,3-tetraethoxypropane, which produces malondialdehyde (MDA), being used as the standard.

Statistical analysis. A statistical evaluation of data was carried out by the use of an analysis of variance (ANOVA) coupled with Tukey’s HSD Multiple Comparison test for the classification of the means. The acceptable level of probability was set at 95%.

RESULTS

Growth parameters

Growth curves for rats fed the various diets are shown in Fig. 1. The weight gained in the first 30 d by the rats of the C-def and C, E-def groups began to be lost after 20 d on the experimental diets. No difference was observed between the control and E-def groups, or between the C-def and C, E-def groups.

The plasma vitamin C level was significantly lower in the C-def and C, E-def groups than in the control and E-def groups (0.9 and 0.5, respectively, versus 7.7 and 7.3 μg/mL plasma, respectively). The plasma vitamin E level was significantly lower in the E-def and C, E-def groups than in the control and C-def groups (0.2 and 0.2, respectively, versus 5.9 and 5.1 μg/mL plasma, respectively). The C-def and C, E-def groups, but not the control or E-def groups, showed symptoms of scurvy, such as bleeding around the nose and convulsions in the legs.

Plasma lipids and lipoprotein cholesterol

The plasma lipid concentrations in each group are shown in Table 2. The concentration of plasma total cholesterol was significantly higher in the C-def and C, E-def groups than in the control group. In the C, E-def group, the total cholesterol level was significantly higher than in the C-def group. In contrast, in the E-def group the total cholesterol and free cholesterol were both lower than in the control group. Plasma triglycerides and phospholipids were also significantly higher than control in both the C-def and the C, E-def groups, but no difference was observed between the control group and the E-def group, or between the C-def group and the C, E-def group.

The distribution of total cholesterol in different lipoproteins is shown in Table 3. In the C-def group, the values obtained for the cholesterol in the Chylomicron(Chyl)+VLDL, LDL, and HDL fractions were about 4.3, 1.8, and 1.2 times the corresponding

![Fig. 1. Effects of deficiency of vitamins C and/or E on body weight in ODS rats. For the first 30 d, all rats were fed the basal vitamin C and E deficient diet and drank distilled water containing 40 mg ascorbate/100 mL. Triangles show the mean body weights for 20 rats throughout this 30 d period. For the next 30 d, the rats were divided into four groups. These groups drank either distilled water (C-def and C, E-def groups) or distilled water containing 40 mg ascorbate/100 mL (control and E-def groups) and were fed either the basal diet (E- and C, E-def groups) or the basal diet containing 10 mg α-tocopherol/kg (control and C-def groups). All rats were maintained by the use of pair-feeding (see Materials and Methods for details). The values are the means for five rats in each group. Open circles, control group; open squares, E-def group; closed circles, C-def group; closed squares, C, E-def group. Asterisks indicate p<0.05 vs. control group.](image-url)
Table 2. Effects of deficiencies of vitamins C and/or E on plasma lipid concentrations in ODS rats.

| Animal       | Total cholesterol | Free cholesterol | Triglycerides | Phospholipids |
|--------------|-------------------|------------------|---------------|---------------|
| Control group| 62.9±5.0a         | 13.3±1.1b        | 56.0±1.8b     | 62.6±1.6b     |
| E-def group  | 49.5±1.2d         | 9.5±0.2c         | 55.9±1.5b     | 57.0±1.0b     |
| C-def group  | 90.4±3.1b         | 21.2±1.4a        | 114.9±3.1a    | 109.2±4.9a    |
| C,E-def group| 101.6±3.0a        | 22.9±0.6a        | 109.7±5.8a    | 108.7±2.7a    |

Values shown are means (mg per 100 mL plasma)±SE.
Means in the same column not sharing a common superscript letter are significantly different (p<0.05).

Table 3. Effects of deficiencies of vitamins C and/or E on total cholesterol distribution in lipoprotein fractions in ODS rats.

| Animal       | Chyl+VLDL | LDL          | HDL          |
|--------------|-----------|--------------|--------------|
| Control group| 7.5±0.8b  | 8.3±0.5c     | 40.1±2.3b    |
| E-def group  | 7.6±0.9b  | 7.3±0.6b     | 32.6±1.5c    |
| C-def group  | 25.2±1.5s | 15.1±1.0s    | 49.8±2.0a    |
| C,E-def group| 24.1±2.7a | 26.1±1.9a    | 48.3±2.2a    |

Values are means (mg cholesterol per 100 mL plasma)±SE.
Means in the same column not sharing a common superscript letter are significantly different (p<0.05).

Table 4. Effects of deficiencies of vitamins C and/or E on TBARS values in the LDL and HDL fractions in ODS rats.

| Animal       | LDL         | HDL         |
|--------------|-------------|-------------|
| Control group| 0.90±0.24c  | 0.77±0.06c  |
| E-def group  | 1.50±0.24b  | 1.59±0.24b  |
| C-def group  | 1.58±0.18b  | 0.99±0.08bc |
| C,E-def group| 2.17±0.49a  | 2.53±0.40a  |

Values are means (nmol malondialdehyde equivalent per mg cholesterol)±SE.
Means in the same column not sharing a common superscript letter are significantly different (p<0.05).

values obtained for the control group. In the C,E-def group, these values were 4.9, 3.2, and 1.2 times those obtained for the control group and about 1.1, 1.7, and 1.0 times those obtained for the C-def group. In contrast, in the E-def group the values obtained for the cholesterol in each of the lipoprotein fractions were similar to the control-group values (except for the HDL levels, which were significantly lower, at about 0.8 times control).

**TBARS value for LDL and HDL fractions**

The TBARS values obtained for the LDL and HDL fractions are shown in Table 4. The values shown for the LDL fraction in the E-def, C-def, and C,E-def groups are all significantly higher than that shown for the control group (1.7, 1.8, 2.4 times, respectively). In the C,E-def group, the value was 1.4 times those obtained for the E-def and C-def groups. The values shown for the HDL fraction in the E-def and C-def groups, but not in the C-def group, are significantly higher than that shown for the control group (2.1, 3.3, 1.3 times, respectively). The value obtained for the C,E-def group was significantly higher than those obtained for the E-def and C-def groups (1.6 and 2.6 times, respectively).

**Electrophoresis**

The results of nondenatured PAGE of plasma lipoproteins are shown in Fig. 2. Increases above control were observed in both VLDL and LDL in the C-def and C,E-def groups. Furthermore, in the C-def and C,E-def groups a band was observed between LDL and HDL2. This lipoprotein was classified in the LDL fraction (d=1.006–1.063) by ultracentrifugation.

The results of SDS-PAGE of the LDL fraction are shown in Fig. 3. Increases above control were observed in apo E in the LDL fraction in the C,E-def group. This change was not observed in the C-def or E-def groups.

**DISCUSSION**

Many studies have suggested a significant effect of vitamin C on plasma cholesterol metabolism. In the present study, we observed a wide range of characteristic effects on lipoprotein properties and metabolism in the vitamin C deficient ODS rat, and some were made even more marked by an additional vitamin E deficiency.

As shown in Table 5, the plasma LCAT activity was significantly lower in each of the deficient groups than in the control group. The activity in the E-def group was the lowest of all groups (significantly lower even than the C,E-def group). No significant difference was observed in LCAT activity between the C-def and C,E-def groups.

**Plasma LCAT activity**

As shown in Table 5, the plasma LCAT activity was significantly lower in each of the deficient groups than in the control group. The activity in the E-def group was the lowest of all groups (significantly lower even than the C,E-def group). No significant difference was observed in LCAT activity between the C-def and C,E-def groups.

As shown in Tables 2 and 3, under the conditions of vitamin C deficiency there were marked increases in plasma VLDL and LDL cholesterol. In contrast, vitamin E deficiency alone led to the level of plasma HDL cholesterol being decreased, but other lipoprotein cholesterol levels did not change. In the group with both vitamin E and C deficiency, the levels of plasma cholesterol, particularly LDL cholesterol, were increased more than in the rats with only vitamin C deficiency. The explanation for our observation that additional vitamin E deficiency led to a further increase in LDL cholesterol in the vitamin C deficient ODS rat is not yet apparent, but one possibility is that the oxidation of LDL may be enhanced when there is a deficiency of these two antioxidative vitamins. Kimura et al. reported that the lipid peroxide concentra-
Fig. 2. Effects of deficiency of vitamins C and/or E on plasma lipoprotein profile, as determined by PAGE. Prestained plasma (A) or LDL fraction (B) (stain, Sudan black B) was applied to 3.75% Tris-HCl-buffered polyacrylamide slab gel with 2.5% stacking gel, and electrophoresis was then performed (see Materials and Methods). LDL fraction (d=1.006-1.063) was fractionated from plasma by ultracentrifugation. The results shown are for 10 μL of sample obtained from control rat, E-def rat, C-def rat, and C, E-def rat, respectively.

Fig. 3. Effects of the deficiency of vitamins C and/or E on apo B in LDL fraction, as determined by SDS-PAGE. LDL fraction (d=1.006-1.063) was fractionated from plasma by ultracentrifugation. SDS-PAGE was performed with 12.5% polyacrylamide slab gels containing 0.1% SDS (see Materials and Methods). The results shown are for 10 μg protein obtained from samples from control rat, E-def rat, C-def rat, and C, E-def rat, respectively. Both side wells contained a mixture of standard proteins: phospholipase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactoalbumin.

Table 5. Effects of deficiencies of vitamins C and/or E on LCAT activity in ODS rats.

| Animal          | LCAT activity (calculated as nmol esterified cholesterol in 1 mL plasma per 1 h reaction time) as mean ± SE. |
|-----------------|----------------------------------------------------------------------------------------------------------|
| Control group   | 95.9 ± 6.0a                                                                                             |
| E-def group     | 66.5 ± 2.1c                                                                                             |
| C-def group     | 74.0 ± 4.5bc                                                                                             |
| C,E-def group   | 75.4 ± 5.3b                                                                                             |

Activity (calculated as nmol esterified cholesterol in 1 mL plasma per 1 h reaction time) is shown as mean ±SE. Means in the same column not sharing a common superscript letter are significantly different (p<0.05).
plasma is associated with the modulation of LDL receptor-mediated clearance. Since HDL has an affinity for LDL receptors about 20 times greater than that of LDL (40), it is possible that the increase in these lipoproteins and LDL in the vitamin C-deficient ODS rat resulted from a down-regulation of LDL receptor expression because of an accumulation of cellular cholesterol. Furthermore, Aulinkas et al. reported that the increased receptor-mediated catabolism of LDL in cultured smooth muscle cells by the supplementation of physiological concentrations of ascorbate (41). Further clarification of the effects of vitamin C deficiency on the regulation of plasma lipoprotein metabolism by influencing LDL receptor-mediated clearance is necessary.

To investigate the effects caused by the deficiency of these vitamins on HDL metabolism, plasma LCAT activity was measured. In plasma, LCAT mostly associates with substrate HDL and produces cholesterol ester by transferring a fatty acid from phosphatidylcholine to the 3-OH base of unesterified cholesterol (42, 43), and a change in HDL cholesterol is associated with LCAT activity (44, 45). The plasma LCAT activity indicates the rate of cholesterol esterification in plasma and the subsequent reverse cholesterol transport. As shown in Table 5, LCAT activity was significantly lower in each of the deficient groups than in the control group. When a common artificial substrate with purified apolipoprotein A-I as a cofactor was used instead of HDL and endogenous apo A-I (see Materials and Methods), no significant difference was observed among the values obtained for the four groups (3.61±0.51, 3.36±0.53, 3.31±0.33, and 3.72±0.39 nmol cholesterol esterified/h/µL plasma in control, E-def, C-def, and C,E-def groups, respectively). Since the HDL cholesterol level was higher in the C-def and C,E-def groups than in the control group (Table 3), the decrease in cholesterol esterification in the plasma in these two groups was not due to decreases in substrate HDL or LCAT enzyme, but to other factors (e.g., oxidative modification of HDL). It has been reported that the oxidation of substrate HDL (46) or the existence of oxidized LDL (47–49) inhibits LCAT activity via a modification of apo A-I (48) or LCAT enzyme (49). The effect on LCAT activity produced by oxidative modification of LDL and HDL because of a deficiency of antioxidative vitamins should be considered in further investigations.

Horio et al. (19) and Kono et al. (21) reported an increase in plasma LDL cholesterol and a decrease in HDL cholesterol in the vitamin C deficient ODS rat. The latter group reported that LCAT activity in the ODS rat was not changed by vitamin C deficiency, despite the low HDL level (21). In the present vitamin C-deficient ODS rats, however, HDL cholesterol was slightly increased and LCAT activity decreased in comparison with the levels in vitamin C sufficient control rats. These discrepancies could be due to the ODS-od/od rats (not ODS+/+ rats) used as a control being maintained through pair-feeding with each diet (to adjust for the decrease in food intake seen in vitamin C deficient animals), since diet restriction decreases the serum cholesterol level (20). In fact, in another experiment we noted that vitamin C sufficient ODS rats maintained with pair-feeding showed decreased HDL cholesterol and increased LCAT activity compared with vitamin C sufficient ODS rats fed ad libitum (0.75 and 1.2 times, respectively).

In conclusion, the effects of vitamin C deficiency on the lipoprotein metabolism become more distinct with greater vitamin E deficiency. It is unclear whether this phenomenon is due to a synergistic action of vitamin C and vitamin E, a type of action noted previously by Igarashi et al. in ODS rats (17), or whether it is a result of actions by these vitamins that are independent of each other. A clarification of the mechanism underlying this phenomenon would be an interesting subject for future investigations.

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