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Studies on the Formation of Aliphatic Aldehydes in the Plasma and Liver of Vitamin E-Deficient Rats

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The effects of vitamin E (E) deficiency on the formation of aliphatic aldehydes in rat plasma and liver were studied. Three-week-old Wistar male rats were fed either an E supplemented diet (2-*ambo*-α-tocopheryl acetate 20 mg/kg diet, designated as E supplemented diet group) or an E deficient diet (E deficient diet group). After 8 weeks, *n*-hexanal and (*E*)-4-hydroxy-2-nonenal (4-HN) in the plasma of the E deficient diet group were found to be 2.0 and 2.5-fold greater than those of the E supplemented diet group, respectively. The contents of aldehydes such as *n*-pentanal, *n*-hexanal, 4-HN in the liver were also significantly higher in the E deficient diet group than in the E supplemented diet group. These results indicate that some aldehydes, arising possibly from lipid peroxides, are produced and detected in the plasma and liver of rats under the condition like E deficiency.

In this study we further found that the activity of the liver aldehyde dehydrogenase (ALDH, EC 1.2.1.3) was significantly changed; 5 and 8 weeks after the start it was lower in the E deficient diet group when compared to that in the E supplemented diet group. The decrease of enzyme activity was related to the increase of aldehydes such as *n*-hexanal in the liver.

The aldehyde increase in the plasma of the E deficient diet group was thought to raise the injury of cells, namely, a strong hemolysis on erythrocytes prepared from the blood of rats fed the E deficient diet.

Keywords vitamin E; lipid peroxidation; *n*-hexanal; (*E*)-4-hydroxy-2-nonenal; aldehyde dehydrogenase

It has been reported that various aliphatic aldehydes are likely to be produced as the result of lipid peroxidation of biological samples such as rat liver microsome.[2)] Among the above aldehydes, some are known to give deleterious effects on enzymes and erythrocytes[3)] which are sometimes much higher than those of lipid hydroperoxide.[4)] It would thus be important and worth studying to know whether these aldehydes could really be produced in the animal body under any circumstance, as there has as yet been no report about their production *in vivo* except in the drastic condition created, for example, by the administration of halogenated hydrocarbon.

Vitamin E (E) is known to act as a strong and efficient antioxidant for protecting lipid peroxidation in the animal body.[5)] It was also reported that thiobarbituric acid reactive substances (TBARS) were increased in the plasma and liver of rats fed an E deficient diet for a long period of time.[6)] Esterbauer[7)] reported that several aldehydes were produced during *in vitro* lipid peroxidation. It would therefore be interesting to determine the aldehydes in the animal body fed the E deficient diet for certain periods of time.

This study was undertaken to examine and analyze aliphatic aldehydes such as *n*-hexanal and (*E*)-4-hydroxy-2-nonenal (4-HN) in the plasma and liver of rats fed the E deficient diet by using the sensitive fluorometric assay system as was previously reported.[8)]

Experimental

Chemicals Reagent grade cyclohexane-1,3-dione (CHD) was purchased from Aldrich Chemical Co. and was purified by recrystalization from reagent grade MeOH. MeOH was distilled after the treatment with reagent grade 2,4-dinitrophenyl hydrazine.

Special reagent grade acetaldehyde was purchased from Merck Co. Aldehyde dehydrogenase (ALDH) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were from Oriental Yeast Co., and reagent grade thiobarbituric acid (TBA) was from Nacalai Tesque Co.

Other chemicals used were of reagent grade quality.

Apparatus High performance liquid chromatography (HPLC) separation and fluorometric measurement (Ex 380, Em 445 nm) were made in Spectra Physics (Type SP-8770) and Hitachi HPLC fluorescence detector (*Model F-1000*), respectively. The column used was ERC-ODS-1262 (100 × 6 mm i.d.; 5 μm particle size, Erma Optical Works Ltd.).

Animals 3-week-old male Wistar rats were obtained from Clea Japan Inc. and were separated into 2 groups. One group (E deficient diet group) was given the basal E deficient diet (less than 0.1 mg E/100 g diet from Oriental Yeast Co.), while another group (E supplemented diet group) was given the basal diet plus 20 mg 2-*ambo*-α-tocopheryl acetate/kg diet as control. Rats were dissected 5 and 8 weeks after starting the experiments.

Analysis Rat livers were removed after perfusion with ice-cold physiological saline. The livers were homogenized in 19 or 9 volumes of 40 mM phosphate buffer (pH 7.4) for the determination of TBARS and aldehydes or for the assay of ALDH (EC 1.2.1.3) activity. The blood of rats was similarly diluted with 19 or 9 volumes of physiological saline and the plasma which was separated after the centrifugation (3 min, 1500 × *g*) was used for the determination of TBARS or aldehydes. TBARS in the plasma and liver were determined by the fluorometric method of Yagi[9)] and the colorimetric method of Masugi *et al.*,[10)] respectively.

E in the plamsa was determined by the fluorometric method of Abe *et al.*[11)]

Aliphatic aldehydes in the plasma and liver were determined by CHD-HPLC fluorometric assay. The details of the method have been described in our previous reports.[8)]

ALDH activity in 10% liver homogenate was measured using acetaldehyde as the substrate.[12)]

Erythrocytes were prepared from the blood of the rats fed for 5 weeks with the E deficient or the E supplemented diet. The hemolysis test of the erythrocytes was performed by the method of Ikehata and Sugiyama[13)] using diarlic acid as the hemolytic reagent.

Results

Effects of E on TBARS in the Plasma and Liver Table I shows E and TBARS in the plasma and/or liver of rats fed the E deficient or the E supplemented diet for 5 and 8 weeks. The amount of E in the plasma of the E deficient diet group was 79% and 60%, respectively, compared to that of the E supplemented diet group after 5 and 8 weeks of feeding. In contrast, TBARS in the plasma of the E deficient diet group increased and the content was significantly ($p<0.001$) higher than that of the E supplemented diet group after 8 weeks. The significant increase of TBARS was also observed in the liver ($p<0.05$) of the E deficient diet group after 8 weeks of feeding and the level was 1.8-fold greater than that of the E supplemented diet group.

Effects of E on the Production of Aliphatic Aldehydes in

TABLE I. E and TBARS in the Plasma and Liver of Rats Fed the E Deficient or Supplemented Diet after 5 and 8 Weeks

	5 weeks feeding		8 weeks feeding	
	E supplemented group	E deficient group	E supplemented group	E deficient group
Plasma				
α-Tocopherol (mg/dl)	0.14±0.02	0.11±0.02[a]	0.15±0.02	0.09±0.02[b]
TBARS (nmol/ml)	2.96±0.05	3.34±0.43	2.30±0.13	3.11±0.15[b]
Liver				
TBARS (nmol/g)	121.1±11.5	136.5±8.5	122.4±54.5	220.2±88.1[a]

a) $p<0.05$, *b*) $p<0.001$. $n=6$.

TABLE II. Aliphatic Aldehydes in the Plasma and Liver of Rats Fed the E Deficient or Supplemented Diet after 5 and 8 Weeks

	5 weeks feeding		8 weeks feeding	
	E supplemented group	E deficient group	E supplemented group	E deficient group
Plasma				
n-Propanal	2.86±0.15	3.01±0.46	2.98±0.66	4.22±1.16
n-Pentanal	5.25±1.22	5.85±1.64	5.72±2.64	9.95±7.45
n-Hexanal	1.03±0.59	1.67±0..39	1.44±0.61	2.89±0.76[b]
4-HN	0.86±0.21	1.11±0.27	0.80±0.20	2.00±1.00[a]
Liver				
n-Propanal	10.34±2.01	12.58±1.95	11.60±1.38	14.80±0.08[c]
n-Pentanal	1.39±0.45	2.07±0.29[a]	1.41±0.05	3.78±0.12[c]
n-Hexanal	3.23±0.15	5.24±0.48[c]	4.26±1.82	6.26±0.18[a]
4-HN	2.82±0.53	4.31±0.29[c]	3.17±0.69	5.49±2.12[a]

The values are mean±S.D. (nmol/ml plasma or g liver). *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$. $n=6$. In plasma and liver, contents of *n*-butanal, *n*-octanal, *n*-nonanal, and *n*-decanal were trace, and they showed no significant difference between two groups.

the Plasma and Liver Table II shows aliphatic aldehydes in the plasma and liver of rats after 5 and 8 weeks of feeding. The amounts of 4-HN and *n*-alkanals in the plasma of the E deficient diet group increased slightly after 5 weeks of feeding, though not significantly, as compared to the aldehydes of the E supplemented diet group. After 8 weeks of feeding, the increases of *n*-hexanal ($p<0.01$) and 4-HN ($p<0.05$) in the plasma of the E deficient diet group were statistically significant as compared to those of the E supplemented diet group. Other aldehydes showed no significant increase in the E deficient diet group, though, in another study, we found significant increases of the aldehydes such as *n*-propanal, *n*-butanal, and *n*-decanal in the plasma of rats fed the E deficient diet for 73 d. The contents of *n*-pentanal ($p<0.05$), *n*-hexanal ($p<0.001$), and 4-HN ($p<0.001$) in the liver of the E deficient diet group after 5 weeks were significantly higher than those of the E supplemented diet group (Table II). Furthermore, *n*-propanal ($p<0.001$), *n*-pentanal ($p<0.001$), *n*-hexanal ($p<0.05$), and 4-HN ($p<0.05$) also showed significant increases in the E deficient diet group after 8 weeks. Such findings demonstrated for the first time that some aliphatic aldehydes could be produced easily in rat plasma and liver under E deficiency.

Correlation between TBARS and Aldehyde in the Plasma and Liver As shown in Fig. 1, a significant correlation was found in the liver between TBARS and 4-HN, or *n*-hexanal after 8 weeks of feeding. In the plasma, weakly positive correlations were observed between TBARS and 4-HN, or *n*-hexanal ($\gamma=0.680$, 0.661 respectively).

Effects of E on ALDH Activities in the Liver ALDH activity is known to be high in rat hepatocyte as compared to the plasma.[14] The activity in the liver of the E deficient diet group decreased to 93% ($p<0.05$) and 68% ($p<0.05$) after 5 and 8 weeks, respectively, compared to that of the E supplemented diet group (Table III). A significantly close and negative correlation ($p<0.01$) between *n*-hexanal and

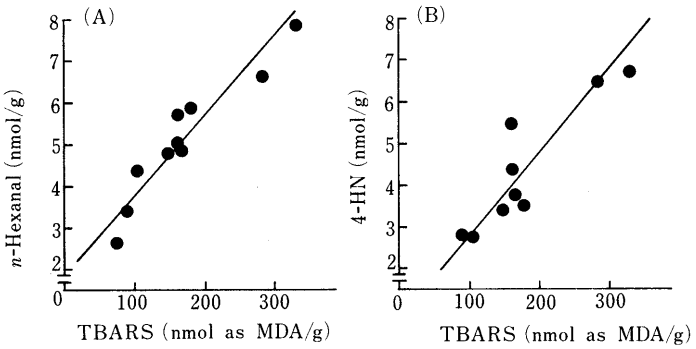

Fig. 1. Correlation between TBARS and Aldehyde in the Liver of Rats Fed the E Deficient or Supplemented Diet after 8 Weeks

A, correlation between TBARS and *n*-hexanal, $r=0.97$ ($p<0.01$); B, correlation between TBARS and 4-HN, $r=0.93$ ($p<0.01$).

TABLE III. ALDH Activity in the Liver of Rats Fed the E Deficient or Supplemented Diet after 5 and 8 Weeks

	ALDH (unit/mg protein)	
	5 weeks feeding	8 weeks feeding
E supplemented group	13.28±0.38	13.04±2.14
E deficient group	12.48±0.52[a]	8.90±3.15[a]

a) $p<0.05$. $n=6$.

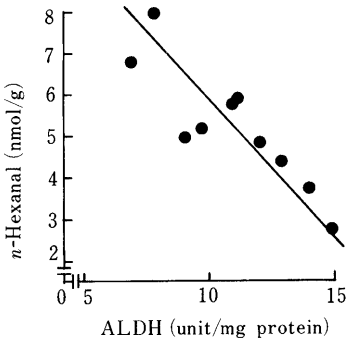

Fig. 2. Correlation between ALDH Activity and *n*-Hexanal in the Liver of Rats Fed the E Deficient or Supplemented Diet after 8 Weeks
$r=0.88$ ($p<0.01$).

TABLE IV. Correlation between Dialuric Acid Induced-Hemolysis and TBARS, or Aldehydes in the Plasma of Rats Fed the E Deficient and Supplemented Diet after 5 Weeks

		Correlation coefficient
Hemolysis *vs.*	TBARS	0.440
	n-Propanal	0.197
	n-Pentanal	0.261
	n-Hexanal	0.720[a]
	4-HN	0.658[a]

a) $p<0.05$. $n=6$ in each group. The lysis of 2.5% erythrocytes in 0.02 M phosphate buffer (pH 7.4) was carried out by the addition of 0.01% diarlic acid followed by incubation at 37 °C for 30 min. The hemolysis of the erythrocyte suspensions was determined from OD at 540 nm of the reaction mixture. The values in this table were calculated from the correlation between the hemolysis and various lipid peroxidation-breakdown products.

ALDH activity was observed in the liver of rats after 8 weeks of feeding, as shown in Fig. 2, though there existed no significant correlation between 4-HN content and ALDH activity. These results suggest that the increase in *n*-hexanal might be accompanied, at least in part, by the decrease of ALDH activity in the liver of the E deficient diet group.

Correlation between the Hemolysis and Some Aldehydes Detected in the Plasma Lysis of the erythrocytes from the rats of the E deficient diet group was observed in approximately 95% of the samples by the addition of dialuric acid, which is in good comparison with only 2% in the E supplemented diet group after 5 weeks of feeding. A significantly large difference was found between the two groups ($p<0.001$). Table IV shows the correlation coefficients between dialuric acid induced-hemolysis and TBARS or aldehydes in the plasma. Although no significant correlation existed between the hemolysis and TBARS, a significantly high and positive correlation was observed between the hemolysis and 4-HN ($p<0.05$), or *n*-hexanal ($p<0.05$).

Discussion

Benedetti *et al.*[15] showed that various aldehydes including *n*-hexanal, *n*-pentanal, and 4-HN were produced from rat liver microsome when they were incubated with adenosine diphosphate-Fe^+ or NADPH-Fe^+. It is of interest to examine whether these aliphatic aldehydes are formed in the animal body when they are kept under coditions susceptible to lipid peroxidation. Dillard *et al.*[16] reported that a small amount of *n*-hexanal was detected in expired gas (under *in vivo* lipid peroxidation) in E-depleted, iron-loaded rats. Benedetti *et al.*[17] also reported that the administration of CCl_4 (400 mg/100 g body weight) into rats increased 4-HN in the liver microsomal fraction. These two studies, however, were carried out under fairly drastic conditions.

Several investigators have pointed out that low E and high TBARS contents have frequently been observed in the blood of experimental animals or patients suffering from atherosclerosis,[18] diabetes,[19] heart disease,[20] and stroke.[21] In the present study, we demonstrated by HPLC-fluorometric assay[8] that some aliphatic aldehydes as lipid peroxide break down products were produced in the plasma and liver of rats fed the E deficient diet. In the liver of the E deficient diet group some aldehydes, such as *n*-hexanal and 4-HN, increased significantly though there was no significant increase of TBARS after 5 weeks of feeding. Esterbauer[7] reported that *n*-hexanal and 4-HN were produced during the course of the autoxidation of authentic unsaturated fatty acids such as linoleic acid. This finding may suggest that lipid peroxidation *in vivo* occured as early as 5 weeks after the start of feeding the E deficient diet and aldehydes arising from lipid peroxides are more sensitive marker substances than TBARS.

It was reported by some investigators that aliphatic aldehydes were able to react with TBA reagent and produced yellow pigments.[22] Therefore, it is thought that TBARS mostly consist of lipid peroxides in themselves, though TBARS include these aldehydes in part.

It would be possible to assume that the increases of aliphatic aldehydes in the liver of the E deficient diet group would be due to the decrease of the metabolic activities of the aldehydes. ALDH is known to be localized in various organs, especially in the liver, and could oxidize C_3—C_{10} alkanals.[14] In this study, we found the negative correlation between *n*-hexanal and ALDH activity in the liver after 8 weeks of feeding. Then ALDH activities may be related to *n*-hexanal contents in the liver. However, 4-HN had no such correlation with ALDH activity. It may partly be explained on the basis that the metabolic pathway of 4-HN is different from *n*-alkanals and gets glutathione conjugation[2a,23] soon after it is formed. In the case of plasma, there was no significant correlation between aldehydes and TBARS, or aldehydes and E. Plasma aldehydes may be released from tissues such as the liver. It is important to examine the origin of the plasma aldehydes and their metabolic pathway, however, we now have little data and information to detect the origin.

Aliphatic aldehydes, especially 4-HN, are known to have high reactivity to SH- and amino-group of the cell constituents and exert deleterious effects on enzymes[13,15a] and nucleic acids.[24] Some aldehydes produced under E deficiency may result in various tissue damages such as the hemolysis of erythrocytes as seen in E deficient animals. It has been reported that 4-HN and the mixture of hydroperoxyalkenals produced from autoxidized methyl linoleate showed a strong hemolytic effect on rat erythrocytes.[2a,25] As seen in Table IV, dialuric acid induced hemolysis of rat erythrocytes under E deficiency were closely correlated with increased plasma aldehydes such as *n*-hexanal and 4-HN.

It may thus be possible that the modifiifactory changes of rat erythrocyte membranes by *n*-hexanal and 4-HN would

be responsible, at least in part, for the facility of dialuric acid induced hemolysis which has long been known and used for the test of E deficiency in experimental animals.

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