Changes in the concentrations of vitamin E analogs and their metabolites in rat liver and kidney after oral administration

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(Received 28 August, 2014; Accepted 16 September, 2014; Published online 20 January, 2015)

Vitamin E analog, such as α- and γ-tocopherol, can undergo α-oxidation without cleavage of the chroman ring, and this pathway is responsible for generation of the major urinary vitamin E metabolite, carboxyethyl hydroxychroman. However, it is still unclear how carboxyethyl hydroxychroman is changed in various tissues after vitamin E intake. We therefore investigated changes in the concentrations of α- and γ-tocopherol and their metabolites in rat liver and kidney. The concentration of α-tocopherol in rat liver increased until 6 h after oral administration, and then decreased. The change in the concentration of α-carboxyethyl hydroxychroman in rat liver in the α-Toc group slowly increased until 12 h after oral administration. Cytochrome P450 3A1 mRNA expression significantly increased from 12 h after the start of α-tocopherol administration. The change in the concentration of γ-carboxyethyl hydroxychroman in rat liver in the γ-Toc group markedly increased until 12 h after oral administration. On the other hand, γ-carboxyethyl hydroxychroman in rat kidney showed greater accumulation than α-carboxyethyl hydroxychroman from 3 h to 24 h after oral administration. From these results, we considered that γ-carboxyethyl hydroxychroman formed in the liver continues to be released into the bloodstream and is transported to the kidney rapidly.

Key Words: Vitamin E metabolism, CEHC, CYP3A, rat liver, rat kidney

Vitamin E has eight different naturally occurring forms: four tocopherols (α-, β-, γ-, δ-Toc) and four tocotrienols (α-, β-, γ-, δ-Toc-3), among which α-Toc, in particular, is known to be a major antioxidant for protection of cellular membranes.1) However, some studies of non-α-Toc are conducted activity recently. Particularly, we focus on the “tocotrienol” from past reports. We previously reported the effect of γ-tocotrienol on rat primary hepatocytes. Form these reports, we clarified that γ-tocotrienol can suppress the inflammation in rat liver,2) and then we considered the suppressible effect may happen via the improvement of ER stress.3) Therefore, we suggested that γ-tocotrienol may prevent steatohepatitis. But, it is not clear how tocotrienol oneself changes in liver when it showed a positive effect. Moreover, it is not clear what an active form of vitamin E is.

Vitamin E analogs absorbed from the small intestine are transported to various tissues with lipoprotein. Those incorporated into the liver are discriminated into α-Toc and non-α-Toc by α-TTP,4) and then α-Toc is secreted into the bloodstream with VLDL. Any of either excess α-Toc or non-α-Toc is rapidly metabolized, excreted in urine5) or bile.6)

When vitamin E analogs are metabolized, it has been suggested that they first undergo α-oxidation by cytochrome P450 (CYP) enzyme, followed by degradation of the side chain by β-oxidation. Finally, they are converted to the CEHC form and excreted into urine. Although the CEHC form is the main metabolite of vitamin E, CEHC—particularly γ-CEHC—has also been reported to have some physiological functions, for example as a natriuretic hormone5,8) and an antioxidant.9) Accordingly, we have considered that CEHC may be the main active form of vitamin E, especially non-α-Toc, in vivo. In fact, we have reported previously that γ-Toc10,11) and γ-Toc-312) accelerate sodium excretion in rats receiving a high-NaCl diet. At 6 to 12 h after administration of γ-Toc-3, the content of sodium in urine was significantly higher than that in rats administrated a placebo, and during the same period, the content of γ-CEHC in urine was significantly higher in rats administrated γ-Toc-3 than in those administrated the placebo.10) On the basis of these results, we speculated that γ-CEHC, an active metabolite of γ-Toc, facilitates the excretion of excessive sodium in vivo. To confirm whether CEHC is indeed the main active form of vitamin E, we think that it will be necessary to clarify the distribution of CEHC in vivo and it has to define the distribution of CEHC in detail if CEHC is a true active form of vitamin E in vivo. In regards to the distribution of CEHC, Uchida et al.13) reported that the distribution of α- or γ-CEHC in rats administrated α-Toc, γ-Toc or ToC3. However, it does not become clear about the changes in the concentrations of tocopherol and CEHC in rat tissues, especially liver or kidney, in the short time.

On the other hand, we established a method for determination of CEHC in biological specimens (rat plasma, urine and bile) using HPLC-ECD.6) However, other than plasma and urine, we have not yet examined whether this method can be used to reliably analyze the CEHC content of individual tissues.

In the present study, therefore, we modified this method to determine CEHC analogs in various tissues, and then investigated the changes in the concentrations of vitamin E analogs (α-Toc and γ-Toc) and their metabolites (α-CEHC and γ-CEHC) in various rat tissues, especially liver and kidney. Moreover, we examined the relationship with the expression of CYP mRNA.

Materials and Methods

Materials. α-CEHC and γ-CEHC were donated by Eisai Co. (Tokyo, Japan).

Animals. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals at Kanagawa Institute of Technology. Male Sprague-Dawley strain rats (5 weeks of age, n = 56) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were initially fed a basic diet...
for three days to allow them to adapt to the new environment. The rats were then fed a diet deficient in vitamin E for 4 weeks. The diet was composed of 40.0% α-cornstarch, 20.0% vitamin-free casein, 25.2% sucrose, 5.0% cellulose, 3.5% mineral mixture, 1.0% vitamin E-free vitamin mixture, 0.3% L-cysteine, 0.014% t-butyldihydroquinone and 5.0% vitamin E-free corn oil. The animals were housed individually in cages at 23 ± 2°C and 55.5 ± 5% humidity under a 12-h light/dark cycle. The feed and water were supplied ad libitum.

**Experimental protocol.** The rats were divided into two groups after 12 h of fasting: a α-Toc group and a γ-Toc group, which were given 10 mg of α- or γ-Toc in stripped corn oil. At 3, 6, 12, 18 and 24 h after oral administration, the rats in each group were killed under diethyl ether anesthesia, and arterial blood and each tissue were removed for analysis. Rats killed at 0 h group were killed rapidly after administration of only stripped corn oil. The plasma was separated from blood cells by centrifugation at 3,000 rpm for 10 min. All tissues were immediately stored at −80°C until analysis. Liver and kidney samples to be used for RNA isolation were soaked in RNA later® solution immediately and stored at −80°C.

**Extraction of vitamin E analogs from rat tissues.** Measurement of vitamin E analogs in each tissue was performed using Ueda’s method. A 0.1 g sample of each organ was homogenized with 0.9 ml of 0.9% NaCl (wt/vol) solution. The homogenate solution (0.2 ml) was then pipetted into a 10 ml centrifuge tube, and 0.2 ml of 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC, 0.9 ml of 0.9% NaCl (wt/vol) solution) and 3.0 ml of 10% ethyl acetate/n-hexane (2:8) was added to each tube with stirring. After addition of 0.2 ml of KOH solution (60%, wt/vol) to each tube, the contents were saponified at 70°C for 1 h under N2 gas. After methylation, the sample tubes were cooled in ice water, 6 ml of 1% NaCl (wt/vol) solution was added to each tube, and the medium was shaken vigorously with 3 ml of n-hexane containing 50 μg/ml BHT for 1 min. This mixture was then centrifuged at 3,000 rpm for 5 min, and 2 ml of the upper layer was collected and evaporated. The residue was dissolved in 100 μl of 41% acetonitrile/water containing 50 mM sodium perchlorate for determination by HPLC.

**Chromatographic apparatus and conditions for CEHC analysis.** The HPLC system consisted of a Shimazu LC-10AT Intelligent HPLC pump, a Shimazu SIL-10AD Intelligent Autosampler, a Shimazu CTO-10AS Column Oven, a Shimazu CBM-20A communication bus module, and a Shimazu DGU-20A3 degasser. The electrochemical detector used a ESA CoulChem II applying a potential of 0.6 V. The analysis of α- and γ-CEHC was performed at 35°C using CAPCELL PAC C18 MGI (5 μm, 250 × 4.6 mm i.d.; Shiseido Inc., Tokyo, Japan). The mobile phase was acetonitrile/water (41:59, vol/vol) containing 50 mM sodium perchlorate (pH was adjusted to 3.6 with acetic acid) at a flow rate of 0.8 ml/min.

**Measurement of CYP mRNA expression in liver and kidney.** Total RNA was extracted from rat liver and kidney using RNA iso. The quantity and purity of the RNA were determined from the absorbance at 258/280 nm. Total RNA was reverse-transcribed into cDNA using a high-capacity RNA-to-cDNA kit in accordance with the manufacturer’s protocol. A 7,500 Fast Real-Time PCR system and a real time PCR kit (TaqMan® Gene Expression Assays, Applied Biosystems Japan Ltd., Tokyo, Japan) were employed based on the manufacturer’s instruction. β-Actin was used as an internal control. The contents of the primer/probe mixture of CYPs and β-actin are shown in Table 2. The PCR run consisted of an initial denaturation at 95°C for 20 s, 40 cycles of denaturation at 95°C for 3 s, and annealing/elongation at 60°C for 30 s.

**Statistical analysis.** All data were expressed as the mean ± SD. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni or Dunnett post-hoc test. Statistical analyses were performed using SPSS for Windows (Tokyo, Japan). Differences were considered significant at *p*<0.05.

| Table 2. Primer probe mixture of CYPs and β-actin |
|---|---|
| **Assay ID** | **Refseq** |
| Rat CYP3A1 | Rn0362228_ml MN_013105.2 |
| Rat CYP3A2 | Rn00756641_ml MN_153312.2 |
| Rat CYP4F1 | Rn00571492_ml MN_019623.2 |
| Rat β-actin | Rn00667869_ml MN_031144.2 |

**Table 1. The analytical recoveries of α-CEHC, γ-CEHC and Trolox from each tissue**

| Tissue    | α-CEHC (CV%) | γ-CEHC (CV%) | Trolox (CV%) |
|-----------|--------------|--------------|--------------|
| Liver (n = 8) | 98 ± 4% (4) | 99 ± 7% (7) | 99 ± 2% (2) |
| Kidney (n = 4) | 92 ± 4% (2) | 91 ± 3% (3) | 96 ± 1% (1) |
| Brain (n = 5) | 97 ± 2% (2) | 100 ± 3% (3) | 100 ± 3% (3) |

Values are mean ± SD.
Results

The concentrations of vitamin E analogs and their metabolites in various tissues at 24 h after oral administration. Fig. 1 shows the concentrations of α-Toc, γ-Toc and their metabolites in rat liver, kidney, adrenal gland, brain, heart and plasma at 24 h after oral administration of α-Toc or γ-Toc, respectively. The concentration of α-Toc in all tissues in the α-Toc group was higher than that of γ-Toc in the γ-Toc group (Fig. 1a). On the other hand, no CEHC was detected in rat adrenal gland, brain and heart. Also, the concentration of γ-CEHC in rat liver, kidney and plasma was higher than that of α-CEHC (Fig. 1b). Then, we examined that the changes in the concentrations of α-, γ-Toc and α-, γ-CEHC in rat liver and kidney until 24 h after the start of oral administration.

Changes in the concentrations of vitamin E analogs and their metabolites in rat liver. The concentration of α-Toc in rat liver in the α-Toc group increased until 6 h after the start of oral administration and then decreased. The concentration of γ-Toc in the γ-Toc group also increased until 6 h after the start of oral administration and then decreased (Fig. 2a). The concentration of γ-CEHC in the γ-Toc group dramatically increased until 12 h after the start of oral administration and then decreased. Furthermore, the γ-CEHC in rat liver was significantly higher than that of α-CEHC from 3 to 12 h after the start of oral administration (Fig. 2b).

Changes in the concentrations of vitamin E analogs and their metabolites in rat kidney. The concentration of α-Toc in rat kidney in the α-Toc group gradually increased until 24 h after the start of oral administration. The change in the concentration of γ-Toc in rat kidney in the γ-Toc group showed a similar trend, but α-Toc was significantly higher than γ-Toc in rat kidney (Fig. 3a). The concentration of α-CEHC in rat kidney in the α-Toc group barely increased until 24 h after the start of oral administration. However, the concentration of γ-CEHC in rat kidney in the γ-Toc group significantly increased continually until 24 h (Fig. 3b). Therefore, we speculated that γ-CEHC released by liver transports to the kidney rapidly via the bloodstream and that γ-Toc was metabolized to γ-CEHC in the kidney at the same time.

Changes in the expression of CYP mRNA in rat liver. Fig. 4a shows the changes in the expression of CYP3A1 mRNA in rat liver until 24 h from the start of α-Toc administration. Expression of CYP3A1 mRNA increased significantly from 12 h after the start of α-Toc administration, and then reached plateau. On the other hand, the expression of CYP3A1 mRNA in liver of rats administered γ-Toc tended to rise in comparison with the leveled at 0 h, but not to a significant degree (Fig. 4b). There was no detection of CYP4F1 mRNA expression in liver of rats administered α-Toc or γ-Toc. Therefore, we considered that CYP4F1 is not related to vitamin E metabolism in rat liver.

Changes in the expression of CYP mRNA in rat kidney. Initially, there was no detection of CYP3A1 mRNA expression in kidney of rats administered α-Toc or γ-Toc. Fig. 5a shows the changes in the expression of CYP4F1 mRNA in rat kidney at 18 h after the start of α-Toc administration tended to be higher than at other time points, but there was no significant difference in the CYP4F1 mRNA expression between all times. Also, the changes in the
expression of CYP4F1 mRNA in kidney of rats administered γ-Toc were similar to those in the α-Toc group (Fig. 5b).

**Correlation between vitamin E analogs and CYP3A1 in rat liver.** Fig. 6a shows the correlation between α-Toc concentration and expression of CYP3A1 mRNA in rat liver. There was no correlation between the α-Toc concentration and CYP3A1 mRNA expression. However, there was a positive correlation between the α-CEHC concentration and expression of CYP3A1 mRNA in rat liver (p<0.05) (Fig. 6b). Therefore, we assumed that CYP3A1 accelerates α-Toc metabolism in rat liver.

**Discussion**

We investigated the changes in concentrations of vitamin E analogs and their metabolites in various tissues of rats administered α-Toc or γ-Toc. Vitamin E is mainly metabolized in the
liver and its carboxy group undergoes α-oxidation. The side chain is then degraded by β-oxidation and vitamin E finally becomes CEHC. Accordingly, CEHC is the main metabolite of vitamin E and has become a marker of vitamin E status in vivo. At 24 h after oral administration of vitamin E, CHECs were accumulated only in liver, kidney and plasma of rats (Fig. 1b). Therefore, we considered that liver and kidney are important tissues for vitamin E metabolism, and we examined the changes in the concentrations of vitamin E metabolites in those organs. The concentrations of both α-Toc and γ-Toc increased in rat liver until 6 h after oral administration, after which the γ-Toc concentration decreased to a greater extent than the α-Toc (Fig. 2a). However, the concentration of γ-CEHC in rat liver had already increased to a greater degree than that of α-CEHC at 3 h after oral administration (Fig. 2b). From these results, we assumed that Toc may be rapidly metabolized to CEHC when the former is absorbed into intestinal cells. Abe et al. reported that the γ-CEHC content was significantly increased in the jejunum at 3 h after administration of γ-Toc or γ-Toc-3. Therefore, we considered that γ-CEHC might be metabolized from γ-Toc in intestinal cells, and then transported to the liver.

On the other hand, the concentrations of both α-Toc and γ-Toc increased in rat kidney from 6 h to 18 h after oral administration (Fig. 3a), suggesting that both α-Toc and γ-Toc accumulated in the kidney after transport from the liver. With regard to CEHC, γ-CEHC showed greater accumulation than α-CEHC from 3 h after oral administration (Fig. 3b). Therefore, we assumed that γ-CEHC is transported from the liver to the kidney. However, as it is unsuitable between the change in the concentration of γ-CEHC in liver and that in kidney, we suggested that γ-CEHC in kidney is not only transported from liver but is also metabolized from γ-Toc extemporarily.

The first step of vitamin E metabolism is α-oxidation, which is dependent on CYP. It is reported that the CYP isoforms involved in vitamin E metabolism are CYP3A[17] and CYP4F[18]. In this study, we investigated the expression of CYP3A1 and CYP4F1 mRNA when rats were administered α-Toc or γ-Toc. CYP3A1 mRNA expression in liver was significantly increased from 12 h to 24 h after administration of α-Toc (Fig. 4a). Conversely, the concentration of α-Toc in the liver decreased from 6 h to 24 h after administration of α-Toc (Fig. 2a). Accordingly, we assumed that α-oxidation of α-Toc may be accelerated by CYP3A1 from 12 h after α-Toc administration. γ-Toc has a great tendency to be metabolized than α-Toc. However, there was no significant difference in the expression of CYP3A1 mRNA after γ-Toc administration (Fig. 4b). Therefore, we considered that α-oxidation of γ-Toc in liver may be involve a CYP isomer, other than CYP3A1. Traber et al. reported that the CYP3A concentration of Ttpa−/−, +/−, +/+ mouse liver was correlated with the concentration of α-Toc, but not that of γ-Toc. Moreover, they observed a strong positive correlation between α-Toc and CYP3A in mouse liver. In the present study, we also found a strong positive correlation between CYP3A1 mRNA expression in rat kidney (Fig. 6b), supporting the contention that α-oxidation of α-Toc in rat liver involves CYP3A1. We also investigated CYP4F1 mRNA expression. CYP4F1 is a one of the CYP homologs preferentially expressed in rat hepatomas, and it has been shown that CYP4F1 is α-hydroxylated to LTB4 and Prostaglandin A1. Therefore, it is considered that rat CYP4F1 has catalytic properties similar to human CYP4F2.

Fig. 5. Changes in the expression of CYP4F1 mRNA in rat kidney. (a), Changes in the expression of CYP4F1 mRNA in rat kidney from 0 to 24 h after α-Toc administration. (b), Changes in the expression of γToc mRNA in rat kidney from 0 to 24 h after γ-Toc administration. The values are mean ± SD for 5–6 for rats.

Fig. 6. Correlation of vitamin E and CYP mRNA expression in rat liver. (a), Correlation of α-Toc concentration and CYP3A1 mRNA expression in rat liver. (b), Correlation of γ-CEHC concentration and CYP3A1 mRNA expression in rat liver.
However, CYP4F1 mRNA was not expressed in liver of rats administered α-Toc or γ-Toc. As it is still unclear whether vitamin E is metabolized to CEHCs in kidney, we investigated changes in CYP mRNA expression in rats administered α-Toc or γ-Toc. However, there was no detection of the expression of CYP3A1 mRNA in the kidney in either rat groups. Sonntag et al. observed α-hydroxylase activity for Toc in rat kidney homogenates and microsomes, suggesting that a vitamin E metabolism system might exist in the kidney. They also assumed that CYP4F1 might be involved in α-oxidation of vitamin E in the liver. Furthermore, Imaoka et al. reported that CYP4F1 was predominantly expressed in rat liver and kidney, especially the kidney. Therefore, we examined changes in the expression of CYP4F1 mRNA in rat kidney. There were no significant differences in the expression of CYP4F1 mRNA in rat liver, but that in rat kidney 18 h after the start of α-Toc administration tended to increase in comparison with other time points (Fig. 5a). Also, the change in the expression of CYP4F1 mRNA in kidney of rats administered γ-Toc was similar to that in the α-Toc group (Fig. 5b). Thus, it remained unclear whether vitamin E is metabolized to CEHCs in the kidney. However, it was clarified that CEHC, especially γ-CEHC, accumulates rapidly in kidney after oral administration. Hereafter, we intend to clarify the mechanism of CEHC transport in a future study.

In summary, we have investigated the changes in the concentrations of Toc, its metabolites and related P450 mRNA expression in rat liver and kidney. CYP3A1 mRNA expression in rat liver decreased from 12 h after the start of α-Toc administration. At the same time, the α-Toc in rat liver decreased and that of α-CEHC barely increased. Therefore, we suggest that α-oxidation of α-Toc occurs until 12 h after oral administration through the action of liver CYP3A1, and then CEHC formed in the liver is released into bloodstream from 12 h. On the other hand, the γ-Toc in rat liver also decreased from 12 h after the start of γ-Toc administration. However, γ-CEHC increased markedly from 3 h after γ-Toc administration. From these results, we consider that α-oxidation of γ-Toc in rat liver begins earlier than that of α-Toc. Moreover, we suggest that γ-CEHC formed in the liver continues to be released into the bloodstream and is transported to the kidney. With regard to CEHC in kidney, we therefore suggest that there are both what was transported from liver and what was metabolized in kidney. Unfortunately, the type of enzyme involved remains unclear and the homologs of Toc-α-hydroxylase that operates in the kidney remains a topic for future study.

Acknowledgments

This work was supported in part by Grant-in-Aid for Scientific Research (KAKENHI) (C) (No.20500726).

Abbreviations

CEHC carboxyethyl hydroxychroman
CYP cytochrome P450
ER endoplasmic reticulum
HPLC high performance liquid chromatography
PMC 2,2,5,7,8-pentamethyl-6-hydroxychroman
Toc tocopherol
Toc-3 tocotrienol
TTP tocopherol transfer protein
VLDL very low density lipoprotein

Conflict of Interest

No potential conflicts of interest were disclosed.

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