

γ -Tocotrienol, a vitamin E homolog, is a natriuretic hormone precursor

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Abstract 2,7,8-Trimethyl-2-(β -carboxyethyl)-6-hydroxychroman (γ -CEHC), a metabolite of γ -tocopherol and γ -tocotrienol, was identified as a new endogenous natriuretic factor. However, γ -tocopherol and γ -tocotrienol, both precursors of γ -CEHC, have never directly been observed to have natriuretic potency. Thus, we investigated whether γ -tocotrienol could cause natriuresis and diuresis in rats. The rats were divided into two groups that were given a control or a high-sodium diet for 4 weeks, and then subdivided into placebo and γ -tocotrienol subgroups given only corn oil-removed vitamin E and oil supplemented with γ -tocotrienol, respectively. After oral administration of three experimental doses, rat urine was collected and γ -CEHC, urine volume, sodium, and potassium content were determined. Only in rats given a high-NaCl diet did γ -tocotrienol accelerate and increase sodium excretion, showing no effect on potassium excretion. Sodium excretion in the high-NaCl group given γ -tocotrienol was 5.06 ± 2.70 g/day, and in the control group given γ -tocotrienol, 0.11 ± 0.06 g/day. Furthermore, γ -tocotrienol affected urine volume in the specific condition of high-NaCl body stores and γ -tocotrienol supplementation. In this study, we found that γ -tocotrienol, one of the natural vitamin E homologs, stimulates sodium excretion in vivo, suggesting that γ -tocotrienol possesses a hormone-like natriuretic function.—Saito, H., C. Kiyose, H. Yoshimura, T. Ueda, K. Kondo, and O. Igarashi. γ -Tocotrienol, a vitamin E homolog, is a natriuretic hormone precursor. *J. Lipid Res.* 2003. 44: 1530–1535.

Supplementary key words γ -CEHC • vitamin E metabolism • natriuresis • urine volume

Vitamin E is the term for a group of tocopherols and tocotrienols that has four homologs (α , β , γ , and δ) differing in the number and position of methyl groups on the chroman ring (1). Tocopherols have saturated tails, whereas tocotrienols have three double bonds in their phytol tails.

Biological activity of these homologs depends on their structures. α -Tocopherol has the highest biological activity of the tocopherols.

Kayden and Traber (2) and Traber et al. (3) clarified that vitamin E homologs (especially α -tocopherol and γ -tocopherol) are equally well absorbed from the intestine, transported by chylomicrons in lymph, and then incorporated into hepatic cells. α -Tocopherol, the only main tocopherol circulating in plasma and detectable in tissues, is preferentially secreted via VLDL. This is mediated by α -tocopherol transfer protein (α -TTP), a cytosolic protein in hepatic cells that specifically discriminates α -tocopherol from other tocopherol homologs (4, 5). Biodiscrimination by α -TTP is related to the bioavailability of each tocopherol, and the relative affinity for tocopherols is as follows: α -tocopherol, 100%; β -tocopherol, 38%; γ -tocopherol, 9%; and δ -tocopherol, 2% (6). Therefore, plasma and tissues are enriched in α -tocopherol, despite higher dietary intakes of γ -tocopherol compared with α -tocopherol. It has also been observed that plasma γ -tocopherol disappears rapidly from the circulation. There is, thus far, no report that γ -tocopherol is localized in hepatic cells after absorption. Kayden and Traber suggested that tocopherols not preferentially transported by α -TTP, such as γ -tocopherol and *SRR*- α -tocopherol, are eliminated into the bile (2).

γ -Tocopherol and γ -tocotrienol are currently discussed as having a role beyond antioxidant. In 1996, Wechter et al. (7) established the structure of a γ -tocopherol metabolite, namely 2,7,8-trimethyl-2-(β -carboxyethyl)-6-hydroxychroman (γ -CEHC; LLU- α), which they found in urine of uremic patients. This compound has no effect on any sodium pump isoform, and is the most potent known inhibitor of the apical 70 pS K^+ channel in the thick ascending limb cells of the kidney's Henle loop (8). Consequently, it

Abbreviations: γ -CEHC, 2,7,8-trimethyl-2-(β -carboxyethyl)-6-hydroxychroman; γ -CEHC-Me, methyl ester of γ -CEHC.

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is assumed to be natriuretic by inhibiting K^+ excretion and K^+ cycling via the $Na^+/K^+/2Cl^-$ cotransporter. Recently, it has been reported that γ -CEHC is produced and excreted into urine not only after ingestion of γ -tocopherol, but also after oral administration of γ -tocotrienol or other vitamin E analogs in rats as well as after γ -tocotrienol supplementation in humans (9, 10). The major pathway by which CEHCs are metabolized from tocopherols and tocotrienols is considered to be ω -oxidation and β -oxidation of the side chain (Fig. 1), forming a major metabolic route of vitamin E homologs in vivo (9–13). However, it is interesting to note that γ -CEHC derived from γ -tocopherol or γ -tocotrienol uniquely exhibits a prolonged natriuresis, in contrast to α -CEHC derived from α -tocopherol (8).

So far, there are no reports of vitamin E affecting urinary sodium excretion. In this study, we investigated whether γ -tocotrienol, one of the vitamin E homologs, can accelerate sodium excretion into rat urine. If γ -tocotrienol were found to play a role in natriuresis, it would be the second example of a vitamin acting as a precursor for a hormone, similar to vitamin D.

MATERIALS AND METHODS

Materials

Standards of γ -CEHC and γ -tocotrienol (Eisai Co. Ltd., Tokyo, Japan) were used, and their purities were 100% and 97.2%, respectively. All agents used in this study were HPLC grade or reagent grade.

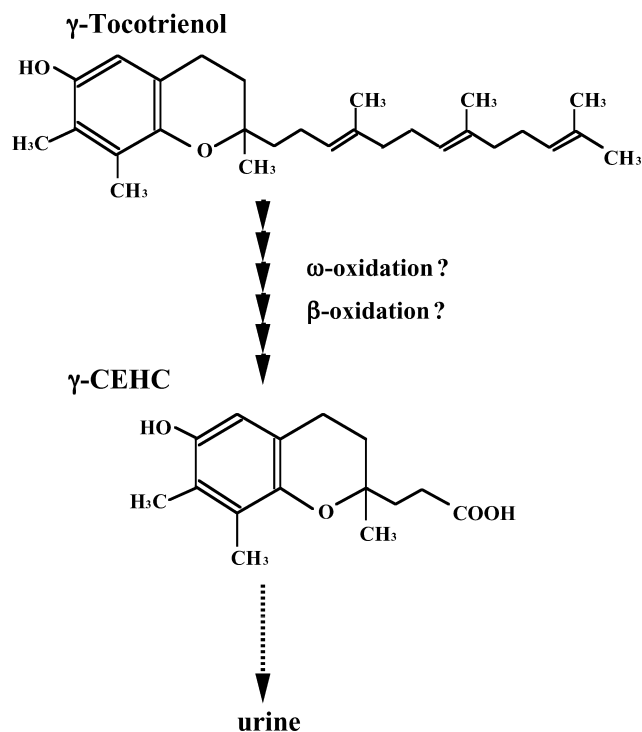


Fig. 1. Possible metabolic pathway of γ -tocotrienol. γ -Tocotrienol is possibly metabolized by ω - and/or β -oxidation to 2,7,8-trimethyl-2-(β -carboxyethyl)-6-hydroxychroman (γ -CEHC), which is then excreted into urine.

Animals

Seven-week-old male Sprague-Dawley rats were purchased from Nippon Clea Co. (Tokyo, Japan) and kept individually in stainless steel cages at $22 \pm 1^\circ C$ and 50% humidity with a 12 h light/dark cycle. The animals were initially fed a commercial diet (CE-2; Nippon Clea Co.) for a week, then divided into two groups. One group was fed a vitamin E-deficient diet, which is an AIN-76 diet modified by Eisai Co. Ltd. (Funabashi Noujou, Chiba, Japan), as a control, and the other group a high-NaCl diet with 5% (w/w) NaCl added to the control diet, for 4 weeks. Feed and water were given ad libitum; however, the settled amounts of diets were 20 g/day over the initial week and 25 g/day over the following 3 weeks; a daily intake of water was observed. To these diets, 10% (w/w) of so-called stripped corn oil (Funabashi Noujou) was added. From this oil, vitamin E was removed by molecular distillation. The composition of the diets is shown in Table 1. This vitamin E-deficient diet was used to induce lower total vitamin E stores before beginning the following experiments.

Experimental procedure and sample collections

Each of the two groups was divided into two subgroups. After 17 h of fasting, each animal of the first subgroup was given 0.5 ml/day of stripped corn oil (Funabashi Noujou) supplemented with 10 mg of γ -tocotrienol by gastric tube over 3 days. The second subgroup received solely stripped corn oil (placebo group). A dose of three daily experimental doses was expected to have a higher natriuretic effect than the natural vitamin. After the last oral administration, the rats were immediately moved into individual metabolic cages, and urine samples were collected in flasks every 6 h over a 24 h period; the flasks were immediately frozen in dry ice during collection. All collected samples were dissolved once for urine volume measurement, followed by immediate lyophilization. The sample powder was stored at $-20^\circ C$ for further analysis.

TABLE 1. Composition of the experimental diet

Dietary Component	Amount	
	Vitamin E-Deficient	Vitamin E-Deficient + NaCl
	g/kg	
Casein, vitamin free	190	190
DL-methionine	3	30
Corn starch	140	140
Sucrose	237	237
Glucose	237	237
Cellulose	47	47
Mineral mixture, AIN-76	33	33
Choline bitartrate	2	2
Corn oil, tocopherol stripped ^a	100	100
NaCl ^b	—	50
	mg/kg	
Thiamin-HCl	5.7	5.7
Riboflavin	5.7	5.7
Pyridoxine-HCl	6.6	6.6
Nicotinic acid	28.5	28.5
D-calcium pantothenate	15.2	15.2
Folic acid	1.9	1.9
D-biotin	0.190	0.190
Vitamin B ₁₂	0.0095	0.0095
Vitamin A	1,140 ^c	1,140 ^c
Vitamin D ₃	950 ^c	950 ^c
Vitamin K	0.0475	0.0475

^a Funabashi Noujou, Chiba, Japan.

^b Wako Pure Chemical Industries, Osaka, Japan; reagent grade.

^c IU/kg diet.

Sample preparation for γ -CEHC HPLC analysis and HPLC conditions

CEHCs are considered to be present as free or conjugated forms *in vivo* (11, 12, 14, 15). Some investigators, therefore, use enzyme assays to measure free CEHCs. Moreover, it is known that CEHCs, especially γ -CEHC, are easily converted to lactones by oxygen (14), making it difficult to accomplish complete quantification. To be able to detect both conjugated and unconjugated CEHC, we established an HPLC method in which methylation yields CEHC-Me, a stable CEHC form suited for extraction and HPLC measurement of both CEHC forms (Fig. 2) (16). The HPLC method applied has been described by Kiyose et al. (16), and is briefly described as follows: the urine powder was dissolved in a defined volume of water, of which an aliquot was removed in another tube with added ascorbic acid and EDTA-2Na. After relyophilization, the aliquot powder was methylated by 3 N methanolic HCl at 60°C for an hour under nitrogen flow. Six milliliters of water was added to the reaction mixture, and extraction was performed with 3 ml of n-hexane. The upper layer was then collected after centrifugation, dried, and finally dissolved in a mobile phase without adjustment of pH scale for HPLC analysis.

HPLC analysis was carried out with an RP-18T C₁₈ column (5 μ m; ϕ 2.0 \times 250 mm, RiX, Saitama, Japan) at 35°C by using a Jasco Gulliver Series PU-980 pump (Jasco Corporation, Tokyo, Japan) and an 860-CO column oven (Jasco). The mobile phase contained 50 mM sodium perchlorate in 45% (v/v) acetonitrile-water adjusted to pH 3.6 with acetic acid at a flow rate of 0.2 ml/min. The effluent was monitored with an electrochemical detector (Nanospace SI-2/3005; Shiseido Co. Ltd., Tokyo, Japan) at +600 mV.

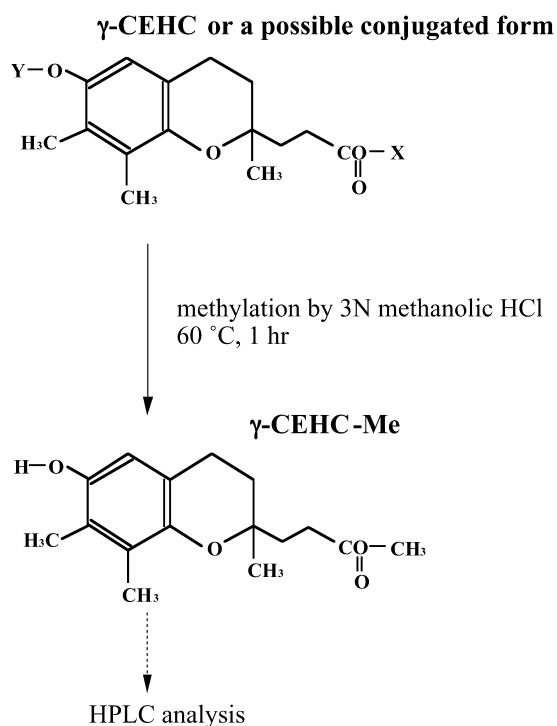


Fig. 2. Structures of conjugated and free γ -CEHC as well as methyl ester of γ -CEHC (γ -CEHC-Me). In this study, γ -CEHC-Me was obtained by treating γ -CEHC with 3 N methanolic HCl and heating at 60°C for one hour. The structure of γ -CEHC-Me was identified using liquid chromatography-mass spectrometry as previously described (16). In this figure, free and conjugated forms are displayed as follows: free, X = H, Y = H; conjugated, X = glucuronate, sulfate or H, Y = glucuronate, sulfate or H, respectively.

Determination of urine volume, sodium, and potassium content in rat urine

Urine volume was measured after urine collection and was standardized by reference to creatinine concentration in urine. Creatinine concentrations in each urine sample were measured by using a kit of Creatinine-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Sodium and potassium content in urine were determined by an atomic absorption spectrometry (Shimadzu AA-610S; Shimadzu Corporation, Kyoto, Japan) after appropriate dilution.

Statistical analysis

All results are expressed as means \pm SD. The significance of the difference between the four experimental groups was evaluated using a multivariate ANOVA (MANOVA). After MANOVA, a Bonferroni-Dunn post hoc test was applied. Analyses were performed using Stat View[®] version 5.0 (SAS Institute Inc., Cary, NC).

RESULTS

Urine volume and excretion of γ -CEHC in rat urine

We investigated the relationship between urine volume and intake of water among all groups (Fig. 3). There was a positive correlation between urine volume and intake of water ($r^2 = 0.65$). It was observed that animals given high amounts of salt had a larger water intake, leading to higher urine output. Urine volumes corrected by creatinine concentration are shown in Fig. 4. In the high-NaCl group given γ -tocotrienol, the level of rat urine volume/creatinine was significantly higher than in the other three subgroups over the 6 h to 12 h period following oral administration ($P < 0.05$). At this time interval, urine volumes were 15.1 ± 3.0 ml and 8.2 ± 4.9 ml in the high-NaCl group with or without γ -tocotrienol, and 2.5 ± 1.0 ml and 2.8 ± 0.4 ml in the control group with or without γ -tocotrienol (data not shown). Urine elimination in the high-NaCl groups was significantly higher compared with

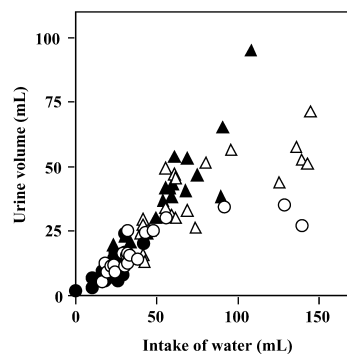


Fig. 3. Correlation between urine volume and water intake per day. Rats were previously fed a vitamin E-deficient diet (control) (circles), or a high-NaCl diet (triangles), for 4 weeks. In each group, the subgroup administered γ -tocotrienol is shown by filled symbols and the placebo group is shown by open symbols. Plots of urine volume against intake of water are displayed for 5 days, including the day just before administration of γ -tocotrienol or placebo, 3 days of administration, and the day after administration. These plots gave a linear relationship at $r^2 = 0.65$.

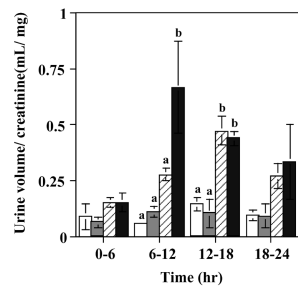


Fig. 4. Changes in urine volume/creatinine. Rats were previously fed a vitamin E-deficient diet (control) or a high-NaCl diet for 4 weeks. In each group, one subgroup was administered γ -tocotrienol, the other placebo. Columns from left to right indicate the following: control + placebo, control + γ -T3, high-NaCl + placebo, and high-NaCl + γ -tocotrienol. Values are means \pm SD of four rats. The significant differences between the experimental groups in every 6 h period were analyzed by a multivariate ANOVA (MANOVA). Bars with different superscript letters are significantly different by a Bonferroni-Dunn post hoc test ($P < 0.05$).

the control groups over the 12 h to 18 h period ($P < 0.05$), as well as up to the 24 h time point.

Large amounts of γ -CEHC in the urine of rats administered γ -tocotrienol were detected in both the control and high-NaCl groups (Fig. 5). In both placebo groups, however, hardly any γ -CEHC was detected. The levels of γ -CEHC excretion in the groups given γ -tocotrienol were significantly higher than in the groups given placebo up to 12 h. γ -CEHC excretion was positively correlated with urine volume in the high-NaCl + γ -tocotrienol group, whereas large amounts of γ -CEHC in the control + γ -tocotrienol group did not affect urine volume (Figs. 4, 5).

Excretion of sodium and potassium in rat urine

The contents of sodium and potassium in rat urine are shown in Fig. 6. In the two high-NaCl groups, urine sodium was excreted to a significantly higher extent in the γ -tocotrienol group than in the placebo group and the

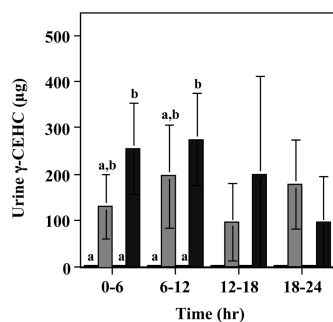


Fig. 5. Changes in γ -CEHC excretion in rat urine. Rats were previously fed a vitamin E-deficient diet (control) or a high-NaCl diet for 4 weeks. In each group, one subgroup was administered γ -tocotrienol, the other placebo. Columns from left to right indicate the following: control + placebo, control + γ -T3, high-NaCl + placebo, and high-NaCl + γ -tocotrienol. Values are mean \pm SD of four rats. The significant differences between the experimental groups in every 6 h period were analyzed by a MANOVA. Bars with different superscript letters are significantly different by a Bonferroni-Dunn post hoc test ($P < 0.05$).

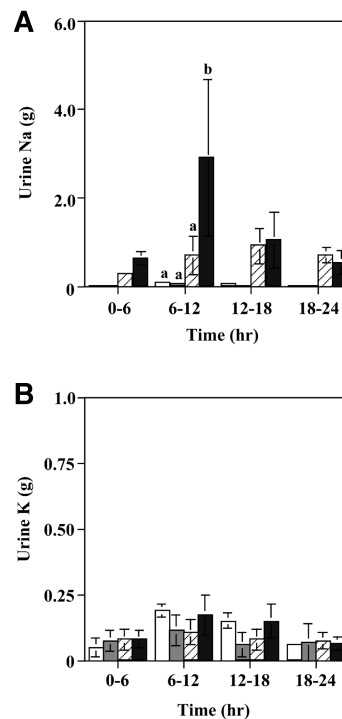


Fig. 6. A: Changes in sodium. B: Changes in potassium excretion in rat urine. Rats were previously fed a vitamin E-deficient diet (control) or a high-NaCl diet for 4 weeks. In each group, one subgroup was given γ -tocotrienol and the other placebo. Columns from left to right indicate the following: control + placebo, control + γ -tocotrienol, high-NaCl + placebo, and high-NaCl + γ -tocotrienol. Values are mean \pm SD of four rats. The significant differences between the experimental groups in every 6 h period were analyzed by a MANOVA. Bars with different superscript letters are significantly different by a Bonferroni-Dunn post hoc test ($P < 0.05$).

control groups during the 6 h to 12 h interval after oral administration ($P < 0.05$) (Fig. 6A). With 2.9 ± 1.8 g, urine sodium was significantly higher in the NaCl + γ -tocotrienol group compared with all other groups. There was, however, no significant difference in sodium excretion between the two control groups and the high-NaCl group given a placebo up to 24 h after oral administration. A large difference in sodium excretion between the control groups and the high-NaCl groups was found in total 24 h sodium excretion after oral administration (data not shown). When comparing daily sodium excretion in all four groups, the high-NaCl group given γ -tocotrienol showed the highest value, amounting to 5.0 ± 2.7 g/day in rat urine. No remarkable difference in potassium excretion was observed in the various groups at any time point (Fig. 6B). Total daily excretion of potassium was observed to be similar, amounting to ~ 400 mg in the various groups, irrespective of feed and dose (data not shown).

Sodium-potassium ratio eliminated in rat urine

γ -CEHC has been known not to affect all sodium pump isoforms and to act as an inhibitor of the apical 70 pS K^+ channel in the thick ascending limb cells of the kidney's Henle loop (8). The ratio of sodium-potassium eliminated in urine is shown in Fig. 7. The time course of the sodium-

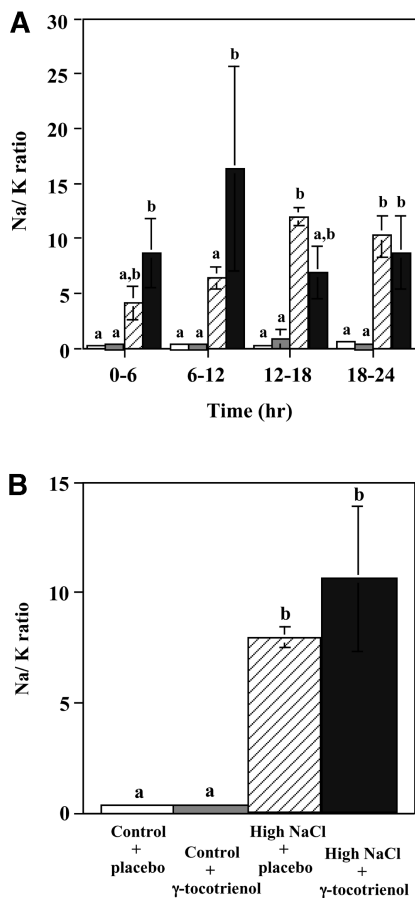


Fig. 7. A: Changes in the ratio of sodium-potassium during the first 24 h divided into 6 h intervals. B: The ratio of total sodium-potassium per day. Rats were previously fed a vitamin E-deficient diet (control) or a high-NaCl diet for 4 weeks. In each group, one subgroup was administered γ -tocotrienol, and the other placebo. Columns from left to right indicate the following: control + placebo, control + γ -tocotrienol, high-NaCl + placebo, and high-NaCl + γ -tocotrienol. Values are means \pm SD of four rats. The significant differences between the experimental groups in every 6 h period and up to 24 h were analyzed by MANOVA. Bars with different superscript letters are significantly different by a Bonferroni-Dunn post hoc test ($P < 0.05$).

potassium elimination is shown in Fig. 7A, while the ratio in a 24 h urine sample is shown in Fig. 7B. In the high-NaCl groups, sodium was excreted into urine to a much higher extent than potassium, especially in the high-NaCl group given γ -tocotrienol. A significant difference compared with the control groups was almost always noted, except in the 12 h to 18 h period after oral administration (Fig. 7A). A constant ratio of sodium-potassium was observed in the control groups up to 24 h after oral administration regardless of the experimental doses (placebo vs. γ -tocotrienol), while a similar ratio of total 24 h sodium-potassium in the two high-NaCl groups was observed (Fig. 7A, B). Total urinary sodium content normalized by total potassium content up to 24 h in the high-NaCl groups was significantly higher than in the control groups (Fig. 7B). This was probably related to the differing dietary intake of salt in the two groups via their experimental feeds.

DISCUSSION

In this study, we investigated whether natriuresis and diuresis were induced by one of the major vitamin E homologs, γ -tocotrienol, in vivo. We report here for the first time that γ -tocotrienol showed a hormone-like function. The daily dietary sodium intake of the experimental animals during the study period was ~ 50 mg in the control groups compared with 1,200 mg in the high-NaCl groups, while the amount of dietary potassium was ~ 70 mg in all groups. Water intake over a 24 h period was related to daily urine excretion in all experimental groups (Fig. 3). Thus, it was assumed that the high urine production was due to the higher water consumption in the high-NaCl groups compared with the control groups (Figs. 3, 4). A large amount of γ -CEHC produced by γ -tocotrienol administration was eliminated in urine of both the control and high-NaCl groups. However, only in the high-NaCl + γ -tocotrienol group was a significant difference detected when comparing these values with the placebo groups up to 12 h after administration (Fig. 5). It can be assumed that the enormous urinary output was due to this significant γ -CEHC excretion in the 6 h to 12 h period, while the large urine excretion in the high-NaCl groups was caused by the high intake of water. In the control + γ -tocotrienol group, no difference of γ -CEHC excretion was seen compared with the high-NaCl group administered γ -tocotrienol, but the urinary output over a 24 h period was far less than in the high-NaCl + γ -tocotrienol group (Figs. 4, 5). This provides evidence that γ -CEHC does not always lead to a higher urine production. Therefore, we conclude that γ -tocotrienol stimulates urinary output only in the presence of a high sodium intake. Furthermore, the urinary γ -CEHC content in the high-NaCl + γ -tocotrienol group was higher than that in the control + γ -tocotrienol group (Fig. 5), suggesting that the production of γ -CEHC from γ -tocotrienol increases when a rise in dietary sodium uptake occurs.

Furthermore, we obtained notable evidence that γ -tocotrienol strongly induced sodium excretion in urine in contrast to potassium excretion when rats were fed a high-NaCl diet, while it is obvious that the presence of high amounts of salt in the body usually stimulate sodium excretion to a higher extent. The above results show that γ -tocotrienol solely accelerates sodium excretion in the presence of high sodium intake (Figs. 6, 7). Interestingly, however, urinary potassium excretion was not influenced by any experimental condition in this study. It is concluded that there may be a relation between sodium excretion and the production of γ -CEHC, functioning as an endogenous natriuretic hormone. Murray et al. (8) reported that γ -CEHC mainly inhibits the 70 pS K^+ channel of the thick ascending limb cells of the kidney's Henle loop and has a function different from that of ouabine, a water-soluble steroid hormone that is an inhibitor of the Na^+/K^+ -ATPase (17). Therefore, our results are consistent with the findings of Murray et al. (8).

Epidemiologic studies suggest that a large intake of dietary salt may pose a considerable risk for hypertension in

developed countries. Tuomilehto et al. reported on the relation between 24 h urinary sodium excretion and cardiovascular risk factors in Finland (18). They suggested that a high salt intake is a predictor of mortality and risk of coronary heart disease, independent of other cardiovascular risk factors, such as high blood pressure. In another study, He et al. recently demonstrated that a relationship between urinary sodium excretion and urine volume exists in hypertensive patients when surplus salt was added to their usual salt intake. The same relationship was also seen in nonhypertensive patients (19). This phenomenon suggests that salt intake is an important factor in controlling urinary volume not only in hypertensive patients but also in normotensive individuals. Sodium excretion was also significantly related to blood pressure in individual subjects (20). Due to these findings, urinary sodium excretion appears to be an important issue in the prevention of hypertension and cardiovascular disease. Consequently, it is likely that a natural compound exists that maintains sodium balance in the body. We propose that γ -tocotrienol, the precursor of γ -CEHC, is an appropriate compound for playing a role of prevention in these diseases.

In summary, γ -tocotrienol, a vitamin E homolog, accelerates sodium excretion and urinary volume in rats given a large sodium intake due to its function as an endogenous natriuretic hormone regulating extracellular volume. We conclude that of the natural vitamin E homologs, γ -tocotrienol, which is metabolized to γ -CEHC, could be considered to be a vitamin functioning as a hormone precursor. Furthermore, we suggest that γ -tocotrienol may prevent hypertension and cardiovascular disease caused by high salt intake. Further studies are needed that focus on the function of vitamin E as a naturally occurring natriuretic hormone. ■■

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