Vitamin C Activity of Dehydroascorbic Acid in Humans —Association between Changes in the Blood Vitamin C Concentration or Urinary Excretion after Oral Loading—

Masaru TSUJIMURA, Shizu HIGASA, Kazuhiro NAKAYAMA¹, Yoshiko YANAGISAWA¹,
Sadahiko IWAMOTO¹ and Yasuo KAGAWA

Kagawa Nutrition University, 3–9–21 Chiyoda, Sakado, Saitama 350–0288, Japan

¹Jichi Medical University, 3311–1 Yakushiji, Shimotsuke, Tochigi 329–0498, Japan

(Received January 29, 2008)

Summary We performed oral loading of AsA or DAsA (1 mmol) in subjects who had consumed a diet low in vitamin C (C) ($C \leq 5$ mg/d) for 3 d before loading, and measured urinary and blood vitamin C. Since the crossover method was used, the same experiment was repeated after an interval of about 1 mo in each subject. The results of the experiment including a total of 17 subjects for 2005 and 2006, were as follows. (1) There were marked individual differences in urinary C excretion. (2) The C level in 24-h urine after C loading did not differ between the two orally administered C forms (AsA and DAsA). (3) C excretion between 0 and 3 h after C loading was significantly higher ($p < 0.05$) for the DAsA group, while those between 3 and 6, 6 and 9, 9 and 12, and 12 and 24 h after C loading were significantly higher ($p < 0.05$ or $p < 0.01$) for the AsA group. (4) The blood C concentration and the increase in C 1 h after C loading were significantly higher ($p < 0.05$ and $p < 0.01$, respectively) in the DAsA than in the AsA group. (5) Evaluation of the association between C metabolism and the single nucleotide polymorphisms of glutathione S-transferase P (GSTP) 1-1 showed a lower urinary C excretion and a significantly lower C level in 24-h urine ($p < 0.05$) after AsA loading, and a significantly lower urinary C excretion between 0 and 3 h after DAsA loading ($p < 0.05$) for the GA heterozygotes than for the AA homozygotes. Considering the activity of C as DAsA in humans, based on urinary and blood C levels after a single loading of C, the utilization of DAsA is equivalent to that of AsA, although the metabolic turnover time is different. The involvement of polymorphisms in the xenobiotic metabolizing enzyme, GSTP1-1, in C metabolism, particularly urinary C excretion, was also clarified. This demonstrates the necessity of considering gene polymorphisms in determining individual C requirements. An abstract of this paper was reported by the Vitamin C Research Committee (Ochanomizu University) in 2007.

Key Words dehydroascorbic acid, ascorbic acid, vitamin C metabolism, glutathione S-transferase P1, polymorphism

Vitamin C (C) is classified as reduced C (L-ascorbic acid, AsA) with acidity, and oxidized C (L-dehydroascorbic acid, DAsA) without acidity. The potency values of both C forms have been considered identical, based on the results of a metabolism study in humans by Tsujimura et al. (1). Based on this understanding, the C value in the Standard Tables of Food Composition, Japan (Composition Tables) (2) has been shown as the total vitamin C value since the 4th edition. The Composition Tables provide basic data on the components of foods eaten daily by Japanese people. At present (2007), the 5th edition is being used.

In recent years, the Vitamin C Research Committee, Vitamin Society of Japan, has planned and performed a re-evaluation of the study performed by Tsujimura et al. (1) because of marked changes in C quantification methods and the recommended C intake in the dietary

intake standards 30 y after their study, as well as the low C activity of DAsA shown by animal experiments using rats lacking C synthesis (3, 4). We have performed metabolism studies in humans to evaluate the C item in the 6th edition of the Standard Tables of Food Composition, Japan, that will be published in the future. In the metabolism study, oral loading of DAsA or AsA was performed in subjects who had had a low-C diet for 3 d, and C in the urine and blood was measured. Urinary C excretion markedly differed among the subjects (5, 6).

C reportedly protects the body from oxidative stress and xenobiotics. Kallner et al. measured the steady-state metabolic turnover and total body pools of C using radioactive C (7). In a comparison between smokers and non-smokers, turnover was 100 mg/d in non-smokers, but increased to 140 mg/d in smokers. DAsA-reducing ability, oxidative stress and polymorphisms in genes associated with detoxification are assumed to be

E-mail: tsuji.m@eiyo.ac.jp

Fig. 1. The scheme of the study. Time "0" was 9 a.m. on the day of the loading experiment, and 1 mmol AsA or DAsA crystals was orally administered at that time. Subjects were healthy female university students (8 females in 2005 and 9 in 2006).

the causes of individual differences in urinary C excretion. The findings reported by Kallner et al. suggest that one cause of individual differences is polymorphisms in xenobiotic enzymes. In this study, we performed C metabolism experiments as reported previously (fiscal 2005), and the results were analyzed in combination with those of the previous report. For typical enzymes associated with xenobiotic metabolism, superoxide dismutase (SOD2 and SOD3) and glutathione *S*-transferase P (GSTP1-1 and GSTP1-2) were selected. Polymorphisms in these enzymes were analyzed in the subjects, and their association with C metabolic findings was investigated.

METHODS

Crystals of each C form. DAsA was purchased from Wako Pure Chemical Industries, Ltd., and AsA from Kanto Chemical Co., Inc. For DAsA, a purity test was performed based on the previous study (8) before this experiment.

Subjects. The subjects were healthy female university students (8 women aged 20–22 y in 2005, and 9 aged 18–22 y in 2006). Consent was obtained from each subject after an adequate explanation of the purpose and procedures of this study before the experiment. This study was performed with the approval of the Medical Ethical Committee, Kagawa Nutrition University and complied with the World Medical Association Declaration of Helsinki (1964, 2000 version).

Metabolism experiment. The scheme of the study is shown in Fig. 1. Because the crossover method was used, the same experiment was repeated after an interval of about 1 mo for each subject. The subjects had a normal diet until 72 h (3 d) before AsA or DAsA loading (9:00) and an experimental diet (low-C diet) thereafter until the discontinuation of the experiment. To fill the in vivo C pool of the subjects, 500 mg AsA was orally administered 1 wk before the loading experiment. On the day of the loading experiment, 1 mmol of AsA (176 mg) or DAsA (174 mg) was orally loaded at 9:00 a.m. Assuming that orally ingested AsA and DAsA are digested and then absorbed as foods, both compounds

were ingested as powder-crystal forms, not aqueous solutions, with 200 mL of commercially available mineral water. Urine was collected for 24 h before the initiation of the low-C diet (normal period) and two 12-h urine samples were collected on the day before the loading experiment (deficiency period). After oral C loading, urine was collected after 3, 6, 9, 12, and 24 h. To prevent the deterioration of collected or stored urine, oxalic acid was added to collected urine to a concentration of 5%. Blood was collected immediately before and 1 and 3 h after C loading.

Experimental diet. A menu with a daily C intake $\leq$ 5 mg was planned. Cooking was performed in the laboratory, and meals were provided for the subjects. During the metabolism experiment period, foods other than those designated (including drugs and supplements) were prohibited. As drinking water, commercially available mineral water was provided, and barley tea or coffee was provided at lunch and supper. For subjects not satisfied with the size of meals, rice or steamed bread not containing C was provided.

C quantification method. Urinary and blood C was diluted with 5% metaphosphoric acid, and C in foods was extracted with 10 volumes of 5% metaphosphoric acid. The dilutions and extracts were combined with dichloroindophenol sodium in order to oxidize AsA to DAsA, and were reacted with 2,4-dinitrophenylhydrazine to produce DAsA osazone. This product was extracted with ethyl acetate and measured by the HPLC method (8). The HPLC conditions were as follows: Column: GL-PACK PARTISIL-5 (4.6 ϕ $\times$ 250 mm, Whatman Co.), mobile phase: *n*-hexane:acetic acid:ethyl acetate=4:1:5 (v/v), flow rate: 1.0 mL/min, detection wavelength: 495 nm, column temperature: room temperature (8).

Calculation of nutritional values. For each nutritional value in the experimental diet, the value listed in the 5th edition of the Composition Tables was used. However, the C content of mushrooms was considered to be 0 mg based on the observed measurement value.

Single nucleotide polymorphism (SNP) analysis. Genotyping of four SNPs, SOD2 Val16Ala (rs4880), SOD3

Table 1. The nutrient values of the low-C diet.

Components	Unit ¹	Year	
		2005	2006
Energy	kcal	1,703±97	1,687±72
Protein	g	71.0±2.8	69.3±5.0
Lipid	g	44.3±11.4	44.2±8.9
Carbohydrate	g	242.9±10.0	242.1±4.4
Ash	g	14.7±0.8	15.0±1.4
Sodium	mg	3,229±294	3,351±495
Potassium	mg	1,923±401	1,883±295
Calcium	mg	552±37	541±72
Magnesium	mg	229±34	226±23
Phosphorus	mg	1,056±32	1,016±67
Iron	mg	8.0±1.7	8.0±1.7
Zinc	mg	8.1±0.8	8.0±0.7
Copper	mg	1.05±0.16	1.05±0.17
Manganese	mg	2.33±0.67	2.40±0.47
Retinol	μg	162±45	148±43
β-Carotene equivalents	μg	957±1,493	1,092±1,405
Vitamin D	μg	8.6±12.8	8.9±12.5
α-Tocopherol equivalents	mg	4.5±0.8	5.0±0.1
Vitamin K	μg	127±149	131±149
Vitamin B ₁	mg	0.98±0.28	0.88±0.32
Vitamin B ₂	mg	1.17±0.15	1.14±0.17
Niacin	mg	16.3±3.5	15.8±4.0
Vitamin B ₆	mg	1.09±0.16	1.07±0.15
Vitamin B ₁₂	μg	7.0±3.8	6.3±4.5
Folate	μg	154±47	167±29
Pantothenic acid	mg	6.12±1.17	5.94±1.05
Ascorbic acid	mg	4.7±0.4	4.9±0.1
Total dietary fiber	g	9.3±2.8	10.3±2.2
Salt equivalents	g	8.3±0.7	8.6±1.2

¹ per 100 g edible portion.

Arg231Gly (rs1799895), GSTP1 Ile105Val (rs1695) and Ala114Val (rs1138272), was performed with the TaqMan genotyping system using an ABI PRISM 7900HT unit (Applied Biosystems, Foster City, CA). The sequences of the TaqMan probes were as follows (polymorphic nucleotides are underlined): for SOD2 Val16Ala, Fam-AGCAGGCAGCTGGCTCCGACT-TAMRA (Ala) and Vic-TGATCTAGCAGGCAGCTGGCTCCGATT-TAMRA (Val); for SOD3 Arg231Gly, Fam-GATCAG GCACTCAGAGCGCAAGAATGG-TAMRA (Gly) and Vic-GCACTCAGAGCGCAAGAATCG-TAMRA (Arg); for GSTP1 Ile105Val, Fam-CAGATCTTGGTGTAGATGAGG GAGTTG-TAMRA (Ile) and Vic-TTGGTGTAGATGAGG GAGTGG-TAMRA (Val); for GSTP1 Ala114Val, Fam-ACGATCCACATAGTCATCCTTGCCTAC-TAMRA (Val) and Vic-CACATAGTCATCCTTGCCTGC-TAMRA (Ala).

Statistical analysis. After analysis of variance using Stat View-J4.02 (Macintosh version), multiple comparison was performed by Bonferroni/Dunn.

RESULTS AND DISCUSSION

The mean age, height, body weight, and BMI of the 17 subjects were 21±1 y, 157±6 cm, 53±7 kg, and 21±3, respectively. The calculated nutrient values of

Fig. 2. Total vitamin C in 24-h urine. Refer to Fig. 1 for the period. The AsA group was given 176 mg of AsA during C loading. The DAsA group was given 174 mg of DAsA during C loading. The values are the mean±SD (Normal period, *n*=15; Deficiency and C loading period, *n*=17). No significant differences were noted in C levels in 24-h urine during any period.

Fig. 3. Changes in total vitamin C urinary excretion for each time after vitamin C loading. AsA (176 mg) or DAsA (174 mg) was given at 0 h. The values are the mean±SD (*n*=17). Vitamin C excretion between 0 and 3 h after DAsA loading was significantly higher than after AsA loading (*p*<0.05), and was lower in the other times (*p*<0.05 or *p*<0.01).

the low-C diet are shown in Table 1. The vitamin C value derived from meals/d was 4.7±0.4 mg in 2005 and 4.9±0.1 mg in 2006. For the calculation of nutrient values, "raw" values in the Composition Tables (2) were used for perishable foods. When vegetables containing C were used, they were drained once after boiling, and therefore, the actual C intake may have been lower than the calculated value.

The results of the metabolism experiment for 17 subjects are shown in Figs. 2–4. The C value was obtained as the total amount of C. The amount of C in urine was expressed as mg/each-hour and that in blood as mg/dL of whole blood. The number of subjects included in each result is shown as *n*. Regarding urinary C, DAsA was separately measured as in the previous report (9), but its content in urine was low (0.1–1.2 mg), and the ratio of DAsA to total C content was not markedly different between AsA and DAsA loading. Thus, only the total C content is presented in this report. No significant differences were noted in C levels in 24-h urine during the normal or deficient period, or after loading between the 2 groups. However, individual variation in

Fig. 4. Total vitamin C in whole blood before and after vitamin C loading. The amount of vitamin C in the blood before vitamin C loading was regarded as 0. The bars indicate blood C concentrations (mean $\pm$ SD) and the lines indicate their increments (mean), ($n=17$). The concentration and increase 1 h after C loading was more marked in the DAsA than in the AsA group (* $p<0.05$, ** $p<0.01$).

the excretion levels were 17.6–85.0 mg after AsA loading and 14.1–61.9 mg after DAsA loading, which represent marked individual differences (over 4-fold). Considering individual differences, excreted C level (mg) corrected against excreted creatinine levels (mg) were also compared, but the findings were similar to those for the measured values (C excretion). C excretion after C loading was significantly higher in the DAsA group between 0 and 3 h after loading but significantly higher in the AsA group during the other periods ($p<0.05$ or $p<0.01$). The blood C concentration was compared between the two groups before and 1 and 3 h after loading. The blood C concentration and the increase in C 1 h after loading were significantly higher ($p<0.05$ and $p<0.01$, respectively) in the DAsA than in the AsA group. The results of this experiment suggested a more rapid C intake into the blood and more rapid C excretion into the urine after oral loading of DAsA than AsA. These results are consistent with those reported by Tsujimura et al. (1). The variations in in vivo C movement after C loading may have been due to differences in the transport system. Na-dependent transporters (SVCT1 and 2) are involved in AsA transport, while passive glucose transporters (GLUTs) are involved in DAsA transport (10). These 3 transporters determine the maximum C transport in the human uriniferous tubule (glomerular filtration rates are about 1.54 and 1.39 mg/min/100 mL in males and females, respectively) and the mean renal threshold (about 1.4 mg/dL) (11). Individual differences are also present in renal clearance, but there were no significant differences in the mean urinary volume or urinary creatinine levels between the AsA and DAsA groups, suggesting that it is difficult to explain the large individual differences in C excretion simply by renal clearance.

In contrast to the large individual differences in urinary C levels, the blood C levels (minimum–maximum) are maintained within a relatively narrow range due to homeostasis. Blood C levels were 0.82 ± 0.12 (0.59–1.07) before loading, 0.94 ± 0.14 (0.59–1.13) mg/dL and 1.14 ± 0.15 (0.84–1.38) mg/dL at 1 and 3 h after

AsA loading, respectively, and 1.09 ± 0.16 (0.79–1.34) mg/dL and 1.14 ± 0.12 (0.94–1.34) mg/dL at 1 and 3 h after DAsA loading, respectively, thus showing that the increase in blood C levels after loading of 1 mmol C was only about 0.3 mg/dL. The range of individual differences in this increase in blood C levels were 0.10–0.48 mg/dL at 1 h after loading and 0.16–0.53 mg/dL at 3 h after loading.

Kallner et al. reported that a higher C intake (more than about 40 mg/d) is necessary for smokers to retain an in vivo C storage equivalent to that in non-smokers, thus suggesting that polymorphisms in enzymes related to xenobiotic metabolism may be associated with individual differences in urinary C excretion. Thus, we analyzed polymorphisms in the SOD2, SOD3, GSTP1-1 and GSTP1-2 genes, and investigated their associations based on the findings for C metabolism. SNPs (SOD2, SOD3, GSTP1-1, and GSTP1-2), as genes expected to be associated with C metabolism, were analyzed at the Division of Human Genetics, Jichi Medical University. Polymorphism was observed in SOD2, SOD3, and GSTP1-1 among the subjects. The genotype frequencies of the genes in the 17 subjects were as follows: the SOD2 AA type was detected in 11 (64.7%) and AG type in 6 (35.3%), SOD3 CC type in 16 (94.1%) and CG type in 1 (5.9%), GSTP1-1 AA type in 12 (70.6%) and GA type in 5 (29.4%), and GSTP1-2 CC type in 17 (100%). The genotype frequencies in the subjects were similar to those in 211 healthy Japanese women (6). Since the incidence of SOD3 polymorphism was low, the association between C metabolism and SOD2 as well as GSTP1-1 was analyzed. As a result, no significant association was observed between C metabolism and SOD2 polymorphism. For GSTP1-1 polymorphism, the C level in 24-h urine after AsA loading was significantly higher ($p<0.05$) for the AA genotype (52.3 ± 20.2 mg) than for the GA genotype (30.1 ± 17.6 mg), and that after DAsA loading was slightly higher for the AA genotype (41.2 ± 14.5 mg) than for the GA genotype (26.4 ± 11.2 mg). Figure 5 shows changes in urinary C excretion after C loading according to the polymorphism types.

Fig. 5. Changes in total vitamin C excretion after vitamin C loading according to the GSTP1-1 polymorphism types. AsA (176 mg) or DAsA (174 mg) was taken at 0 h. The values are the mean $\pm$ SD. AA and GA were a genotype polymorphism of glutathione *S*-transferase P1-1. Vitamin C excretion of the AA genotype in each of time was higher than for the GG genotype.

When AsA was orally loaded, the excretion levels for the AA genotype of GSTP1-1 were higher than that those for the GA genotype in all excretion periods, and the excreted C levels in the 6–9 and 9–12 h periods after AsA loading were significantly higher. When DAsA was loaded, the excreted C levels in urine collected at 0–3 h after loading was significantly higher for the AA genotype than for the GA genotype.

This was a single oral load test. As food intake affects urinary C excretion, subjects ingested the same low-C diet beginning 3 d before the loading experiment until test completion. Ingestion of the low-C diet for 3 d reduced the normal-state, and 24-h urinary C excretion ranged from 41.3 ± 23.6 (3.4–85.0) mg to 4.7 ± 1.5 (3.0–9.5) mg. The experimental diet provided may have been appropriate for the C metabolism test. After ingestion of the low-C diet for 3 d, subjects ingested 1 mmol of C crystals with mineral water. With regard to the loading dose, in a previous study performed by Tsujimura et al., C excretion in 24-h urine was measured after the ingestion of 50, 100, 300 and 600 mg of AsA in a preliminary test to determine the loading dose (300 mg) that facilitated investigation of excretion without considering normal value variation, and the dose was set at 600 mg in consideration of safety. However, dietary C intake is recommended at about 75 mg/d in women in their 20s according to the National Nutrition Survey, which suggests that 600 mg is excessive. In the preliminary study performed by Tsujimura et al., the excreted C level in 24-h urine after loading 100 mg of AsA was 23.5 mg, and normal value variation markedly affected excretion at this dose.

We investigated the loading dose based on the results of previous studies, actual C intake, and the recommended intake (100 mg) for adults, and set the dose at 1 mmol. In the single dose test with 1 mmol of C, DAsA was incorporated into the circulation faster than AsA, and the excess was rapidly excreted, but no significant differences were noted in C levels in 24-h urine, thus suggesting that the metabolism and utilization of DAsA are equivalent to those of AsA. It was also confirmed that gene polymorphisms of GSTP1-1, an enzyme related to xenobiotic metabolism, are associated with the marked individual differences in excreted C levels.

However, it is unlikely that individual differences in C metabolism can be explained by polymorphisms of GSTP1-1 alone, and consideration of polymorphisms in other genes, in addition to lifestyle factors (such as smoking or degree of stress), is necessary to determine individual C requirements. Investigation of association with various other polymorphisms may clarify the details of C metabolism in humans. We are also planning to perform a long-term loading experiment involving the ingestion of AsA or DAsA.

Acknowledgments

We express deep gratitude to graduates (2005, 2006) in Bioorganic Chemistry, Kagawa Nutrition University, for their cooperation with this study.

REFERENCES

- 1) Tsujimura M, Watanabe S, Michinaka K, Tokuhisa S, Fujita A. 1972. Metabolism of ascorbic acid and dehydroascorbic acid in man. *Vitamins* **45**: 136–147 (in Japanese).
- 2) The subdivision on resources, The Council for Science and Technology, Ministry of Education, Culture, Sports, Science and Technology, Japan. 2005. Standard Tables of Food Composition in Japan, Fifth revised and enlarged edition. National Printing Bureau, Tokyo (in Japanese).
- 3) Ogiri Y, Fang S, Hayami S, Fujimura A, Yamamoto K, Yaita M, Kojo S. 2002. Very low vitamin C activity of orally administered L-dehydroascorbic acid. *J Agric Food Chem* **50**: 227–229.
- 4) Higasa S, Tsujimura M. 2004. Vitamin C activity of dehydro-L-ascorbic acid in ODS rats. *Vitamins* **78**: 319–325 (in Japanese).
- 5) Tsujimura M, Higasa S. 2007. Vitamin C activity of L-dehydroascorbic acid in human. Time-dependent vitamin C concentration in blood and urinary excretion after the oral load. *Vitamins* **81**: 489–496 (in Japanese).
- 6) Higasa S, Tsujimura M, Hiraoka M, Nakayama K, Yanagisawa Y, Iwamoto S, Kagawa Y. 2007. Polymorphism of glutathione *S*-transferase P1 gene affects human vitamin C metabolism. *Biochem Biophys Res Commun* **364**: 708–713.
- 7) Kallner AB, Hartmann D, Hornig DH. 1981. On the requirements of ascorbic acid in man: steady-state turnover and body pool in smokers. *Am J Clin Nutr* **34**:

- 1347–1355.
- 8) Tsujimura M, Higasa S, Kasai T. 1996. Quantitative determination of total vitamin C in foods by HPLC using 2,4-dinitrophenylhydrazine as the Internal Standard. *Vitamins* **70**: 241–248 (in Japanese).
- 9) Tsujimura M, Higasa S, Aono K, Seki T. 2006. Vitamin C activity of L-dehydroascorbic Acid in human. Time-dependent vitamin C urinary excretion after the oral load. *Vitamins* **80**: 281–285 (in Japanese).
- 10) Wilson JX. 2005. Regulation of vitamin C transport. *Annu Rev Nutr* **25**: 105–125.
- 11) Oreopoulos DG, Lindeman RD, VanderJagt DJ, Tzamaloukas AH, Bhagavan HN, Garry PJ. 1993. Renal excretion of ascorbic acid: effect of age and sex. *J Am Coll Nutr* **12**: 537–542.