Characteristics of Ascorbic Acid Metabolism in Scurvy-Prone Spontaneously Hypertensive Rat, SHR-od

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Summary SHR-od is a novel strain of rat that spontaneously develops hypertension and has a defect of ascorbic acid (AsA) biosynthesis. The osteogenic disorder Shionogi (ODS) rat is normotensive and also unable to synthesize AsA. To investigate whether or not genetic hypertension affects AsA metabolism, we compared the AsA metabolisms of SHR-od and ODS rats. In this study, a physiological dose of AsA equivalent to the AsA requirement in ODS rats was administered to rats intraperitoneally (i.p. group) or orally (oral group). We measured AsA concentrations in the serum, liver, kidney, adrenal glands, and spleen, and the amount of AsA excreted into the urine. At 25 wk of age (hypertensive status), the AsA concentrations of all tissues tested were significantly lower in SHR-od than in ODS rats in both the i.p. and oral groups. In the i.p. group, the amount of urinary AsA in SHR-od was also lower than that in ODS rats. At 4 wk of age (before the onset of hypertension), liver and spleen AsA concentrations in SHR-od were lower than those in ODS rats in both the i.p. and oral groups. Urinary AsA excretion from SHR-od was not different between the two groups. Our data suggest that the requirement for AsA in SHR-od is increased to maintain tissue AsA concentrations equivalent to those in ODS rats, and that a larger part of the AsA administered to rats in this study is degraded in SHR-od as compared to ODS rats.

Key Words ascorbic acid, hypertension, spontaneously hypertensive rat, ODS rat, SHR-od

Ascorbic acid (AsA) acts as a cofactor of some hydroxylases essential for collagen (1, 2) and catecholamine biosynthesis (3). Moreover, AsA is a water-soluble antioxidant and a free-radical scavenger. Therefore, it is estimated that the oxidation and subsequent degradation of AsA are accelerated under oxidative stress. We have reported (4) that treatment with lipopolysaccharide, which is recognized as an experimental model for oxidative stress, causes a marked reduction of tissue AsA concentration, for example, in the liver of osteogenic disorder Shionogi (ODS) rats unable to synthesize AsA, and that the bilirubin oxidation provoked by lipopolysaccharide treatment was suppressed by feeding AsA to ODS rats. These results mean that AsA protects the body and organs against oxidative damage from oxidative stress.

Several studies have shown an inverse correlation between serum AsA concentration and blood pressure in human subjects (5–8) and experimental animals (9). Free-radical production and the oxidation process are involved in several aspects of the pathology of hypertension (10). The spontaneously hypertensive rat (SHR) is an available disease model for human essential hypertension that was established from a colony of Wistar-Kyoto rats (WKY) (11). SHR and WKY are able to synthesize enough AsA in the liver to maintain a high concentration of AsA in various tissues. The cardiac superoxide dismutase activity in SHR was reported to be lower than that in normotensive WKY (12), and the level of superoxide anion in the SHR heart seems to be high. It has been hypothesized that a high level of superoxide causes the cardiac hypertrophy observed in SHR (12). Moreover, the vascular activity of NAD(P)H oxidase, which produces superoxide anion, is reported to be enhanced in SHR as compared to WKY (13). The increased superoxide production caused by NAD(P)H oxidase enhancement may contribute to endothelial dysfunction (14, 15) and vascular hypertrophy (16) in SHR. These results also suggest that the increase in blood and tissue superoxide anion in SHR causes the consumption of physiological antioxidants such as AsA.

We have established a novel strain of SHR, SHR-od (17) with a hereditary defect of AsA biosynthesis through cross breeding with the ODS rat, which is a rat mutant unable to synthesize AsA due to a mutation of the L-gulono-7-lactone oxidase (EC 1.1.3.8) gene (18), and have revealed several pathophysiological characteristics of this unique model. SHR-od is a useful animal model for precisely investigating the interrelationship between hypertension and the AsA metabolism.

The objective of the present study was to examine the
effect of the genetic background for hypertension of SHR on AsA metabolism using SHR-od and ODS rats. We compared the serum, tissue, and urinary levels of AsA between SHR-od and normotensive ODS rats at both 4 and 25 wk of age.

MATERIALS AND METHODS

Animals and diets. SHR-od were drawn from a population maintained in the animal colony of Nagoya University. Male ODS rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). All rats were housed in individual wire screen-bottomed cages in the animal colony of Nagoya University and maintained at 24°C with a 12-h light cycle (lights on from 0800 to 2000). Until 4 or 25 wk of age, rats were allowed free access to drinking water and a stock chow diet (Labo MR-breeder, Nihon Nosan Kogyo, Yokohama, Japan). All procedures were performed in accordance with the Animal Experimentation Guides of Nagoya University.

Experimental design. In experiment 1, male SHR-od and ODS rats 4 wk of age were divided into two groups of four animals each. In one group (i.p. group), rats were intraperitoneally injected with AsA at a dose of 54.6 mg (dissolved in 0.9% saline) per kg body weight once a day for 21 d. In the other group (oral group), rats were orally administered the same dose of ascorbic acid in 0.9% saline by stomach tube (KN-349 01.5 mm, Natsume Seisakusyo, Tokyo, Japan) once a day for 14 d. These treatments were performed at 6 p.m. every day.

In experiment 2, male SHR-od and ODS rats 25 wk of age were divided into two groups of four animals each. In one group (i.p. group), rats were intraperitoneally injected with AsA at a dose of 18.3 mg (dissolved in 0.9% saline) per kg of body weight once a day for 21 d. In the other group (oral group), rats were orally administered the same dose of ascorbic acid in 0.9% saline by stomach tube (KN-349 φ1.5 mm, Natsume Seisakusyo, Tokyo, Japan) once a day for 14 d. These treatments were performed at 6 p.m. every day.

Feeding a diet containing 300 mg AsA per kg prevents the development of scurvy in ODS rats (19) and SHR-od (17). When ODS rats and SHR-od were fed this diet ad libitum at 4 wk of age, their daily AsA intake was measured as 54.6 mg per kg body weight. At 25 wk of age, the daily AsA intake in ODS rats and SHR-od was measured as 18.3 mg per kg body weight. In this study, the daily dose of AsA in each experiment was determined using these results.

All rats were fed a Funabashi-SP diet (Funabashi Farm, Chiba, Japan) during the experimental period. The composition of the diet is as follows: crude protein, 20.8%; crude fat, 4.8%; nitrogen-free extract, 58.2%; crude ash, 5.0%; crude fiber, 3.2%; moisture, 8.0%. Since Funabashi-SP diet contains a negligible amount of AsA, feeding this diet causes scurvy in ODS rats and SHR-od. All rats were allowed free access to the diet and water. Systolic blood pressure was measured by the tail-pulse pick-up method without anesthesia using an automatic blood pressure monitoring system (BP-98A, Softron Co., Ltd., Tokyo, Japan). For collecting urine, rats were placed in metabolic cages for 24 h on day 13 (experiment 1) or 20 (experiment 2). Urine was collected in a 100-mL glass flask containing 10 mL of 5% metaphosphoric acid for the ascorbic acid assay or distilled water for the creatinine assay. Collected urine was filtered with filter paper (ADVANTEC No. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and stored at −80°C until the assay. Rats were sacrificed by decapitation. Blood was collected, and serum was prepared by centrifugation at 1,500×g for 10 min at 4°C. Liver, spleen, kidney, and adrenal glands were quickly removed, and the liver was perfused with 0.9% saline. Then, all tissues were quickly frozen in liquid nitrogen and stored at −80°C.

Determination of tissue, serum, and urinary ascorbic acid concentrations. Tissues stored at −80°C were homogenized in ice-cold 5% metaphosphoric acid and centrifuged at 1,500×g for 10 min at 4°C. In the case of serum, 10% metaphosphoric acid was added at a ratio of 1:1 and the mixture was kept on ice for 10 min. Next, it was centrifuged at 1,500×g for 10 min at 4°C. The ascorbic acid concentrations of the supernatant and filtrated urine were measured by the dinitrophenyl-hydrazine method (20) with a modification in which the oxidation of ascorbic acid was accomplished using 2,6-dichlorophenol-indophenol.

Determination of urinary creatinine level. Urinary creatinine was determined using a commercial kit (Creatinine-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Determination of lipid peroxidation in liver and serum. The degree of lipid oxidation in the liver and serum was measured using the thiobarbituric acid reactive substance (TBARS) assay. The frozen liver was homogenized with 10 vol of ice-cold 1.15% KCl and the TBARS assay was performed using the method of Okawara et al. (21).

Assay for the hepatic GSH-Px activity. The liver was homogenized with 6 vol of ice-cold 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 1 mM dithiothreitol. Then, the homogenate was centrifuged at 10,000×g for 15 min and the supernatant was used for the GSH-Px assay, which was performed using a Glutathione Peroxidase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The protein concentration of the supernatant was determined using a Bio-Rad Protein Assay. Rats were sacrificed by decapitation. Blood was collected, and serum was prepared by centrifugation at 1,500×g for 10 min at 4°C. The degree of lipid oxidation in the liver and serum was measured using the thiobarbituric acid reactive substance (TBARS) assay. The frozen liver was homogenized with 10 vol of ice-cold 1.15% KCl, and the TBARS assay was performed using the method of Okawara et al. (21).

Statistical analysis. All values are presented as means±SE. When variances of each group were equal, the statistical analysis of difference was performed using unpaired Student’s t test (Stat-100, Biosoft, Cambridge, UK). When variances of each group were unequal, the statistical analysis of difference was performed using Welch’s t test (22, 23). Comparisons were made between ODS rat and SHR-od within the i.p. and oral groups. Differences with p<0.05 were regarded as significant.
Ascorbic Acid Metabolism in SHR Unable to Synthesize Ascorbic Acid

Table 1. Body weight and systolic blood pressure.1

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<td>Body weight, g</td>
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<tr>
<td>Initial</td>
<td>79±1</td>
<td>48±4**</td>
<td>78±2</td>
<td>48±7*</td>
<td>306±14</td>
<td>301±9</td>
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<tr>
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<td>151±1</td>
<td>105±6*</td>
<td>140±4</td>
<td>102±10**</td>
<td>323±12</td>
<td>307±10</td>
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<tr>
<td>Blood pressure, mmHg</td>
<td>Initial</td>
<td>107±6</td>
<td>116±3</td>
<td>105±6</td>
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1 Values are means and SE. n=4 (4 wk) or 5 (25 wk).
* Significantly different from the value of ODS rats at the respective administration using Student’s t test (*p<0.05; **p<0.01).
# Significantly different from the value of ODS rats at the respective administration using Welch’s test (#p<0.05; ##p<0.01).

RESULTS

Experiment 1 (at 4 wk of age)

As shown in Table 1, body weight was significantly lower in SHR-od than in ODS rats at 4 wk of age. There were no significant differences in systolic blood pressure between ODS rats and SHR-od during the course of the experiment. Moreover, the differences in the route of AsA administration did not affect the systolic blood pressure of ODS rats or SHR-od.

Serum AsA concentrations were not different between ODS rats and SHR-od in either the i.p. or oral groups (Fig. 1A). In the i.p. groups, kidney and adrenal AsA concentrations were not different between ODS rats and SHR-od (Fig. 1B and 1E). However, in the oral groups, kidney and adrenal AsA concentrations were significantly lower in SHR-od than in ODS rats. Hepatic and spleen AsA concentrations in SHR-od were significantly lower than those in ODS rats in both the i.p. and oral groups (Fig. 1C and 1D).

Urinary excretion of AsA and creatinine is shown in Fig. 2A and 2B, respectively. The amounts of urinary AsA and urinary creatinine were not different between ODS rats and SHR-od in either the i.p. or oral groups. The amount of AsA excreted by SHR-od was 27% of the administered AsA in the i.p. groups and 0.9% in the oral groups.

As shown in Fig. 3A, the hepatic TBARS level tended to be higher in SHR-od as compared to that in ODS rats, but not significantly. Hepatic GSH-Px activity was lower in SHR-od than in ODS rats (significant in the oral group, but not significant in the i.p. group).

Experiment 2 (at 25 wk of age)

At 25 wk of age, body weight was not different between the ODS rats and SHR-od (Table 1). The systolic blood pressure of SHR-od was significantly higher than that of ODS rats. The differences in the route of AsA administration did not affect the systolic blood pressure of either ODS rats or SHR-od.

The serum AsA concentration in SHR-od was significantly lower than that in ODS rats in the i.p. group (Fig. 1).
As shown in Fig. 2A, in the i.p. groups, the amount of urinary AsA from SHR-od was significantly lower (34% of the amount in ODS rats) than that from ODS rats. In the oral groups, as the amount of urinary AsA was markedly low, a difference was not observed between ODS rats and SHR-od. The amount of creatinine was not different between ODS rats and SHR-od (Fig. 2B).

The hepatic TBARS level in the i.p. group was significantly higher in SHR-od than that in ODS rats, but was not significant in the oral group (Fig. 3A). Hepatic GSH-Px activity in SHR-od was significantly lower than that in ODS rats in both the i.p. and oral groups (Fig. 3B).

DISCUSSION

ODS rats with a hereditary defect of AsA biosynthesis are a useful model for determining the requirement for this vitamin under various conditions. In young adult ODS rats, the dietary addition of about 300 mg AsA per kg of diet is sufficient to prevent signs of scurvy and to achieve maximum growth (19). In addition to ODS rats, we (17) established a novel SHR strain unable to synthesize AsA, SHR-od, and reported that the dietary addition of 300 mg AsA per kg of diet is also sufficient to prevent scurvy in SHR-od. SHR-od develops spontaneous hypertension from around 10 wk of age, and the systolic blood pressure of SHR-od reaches 180–200 mmHg at 20 wk of age (17). With the establishment of SHR-od, it has become possible to examine the effects of the genetic background of spontaneous hypertension or the effect of the development of hypertension on AsA metabolism under the physiological intake of this vitamin.

In this study, the AsA dose in each experiment was determined by the calculation of daily AsA intake when SHR-od and ODS rats were fed a diet containing 300 mg AsA per kg of diet. We adopted two ways of administering AsA. The first method was oral administration, which is more physiological, and the other method was i.p. injection. Using i.p. injection, the difference in intestinal AsA absorption between the two strains of rats can be ignored. Moreover, in our experiments, we used SHR-od both before and after they had developed hypertension. As a result, serum and tissue AsA concentrations in the oral group were lower than those in the i.p. group at both 6 and 28 wk of age. A comparison with previous results (24) revealed that the hepatic AsA concentration of 6-wk-old ODS rats in the oral group was lower than that of 6-wk-old ODS rats fed a diet containing 300 mg AsA per kg of diet (58 ± 2 versus 147 ± 2 µg/g). This difference is due to the low absorption rate of AsA in the oral group administered AsA once a day.

At 25 wk of age, the systolic blood pressure of SHR-od was markedly higher than that of ODS rats, and SHR-od had been exposed to hypertension since around 16 wk of age. After the experimental period, the AsA
concentrations in all tissues tested were markedly lower in SHR-od than those in ODS rats in both the i.p. and oral groups. The serum concentration of AsA in the i.p. group also tended to be lower in SHR-od as compared to ODS rats. In the oral groups, there was no difference in serum AsA concentration between the two strains, because the concentrations were markedly low. In a previous study (17), we reported that serum and hepatic concentrations of AsA were also lower in hypertensive SHR-od than those in ODS rats under feeding a diet containing 300 mg AsA per kg ad libitum. AsA itself and the AsA metabolites produced via its degradation pathway were excreted into the urine at a constant rate. In the i.p. groups, the amount of AsA daily excreted into the urine of SHR-od was 34% of that of ODS rats (the difference was significant, p<0.05). There was no difference in urinary amount of AsA between the two strains in the oral groups, because the amount of urinary AsA in these groups was too low.

At 4 wk of age, the serum AsA concentration and urinary excretion of AsA were not different between ODS rats and SHR-od in either the i.p. or oral groups. However, AsA concentrations in the liver, spleen, and adrenal gland in SHR-od were lower than those in ODS rats.

These results suggest that the body pool of AsA is lower in SHR-od, especially after the development of hypertension, than in ODS rats, probably due to the greater degradation of AsA in SHR-od. In future studies, the amount of AsA metabolites in various tissues, serum, and urine should be compared between these two strains. In addition, the turnover rate of AsA in the serum and various tissues should be measured in these two strains.

SHR were derived from WKY, and the hypertension of SHR is controlled and inherited by polygenes (25). Both SHR and WKY are capable of synthesizing sufficient AsA in their liver, and they can maintain high concentrations of AsA in various organs. For instance, the liver concentration of AsA in 25-wk-old SHR and WKY fed an AsA-free diet is 170–270 μg/g. Although data are not shown, no differences in liver, spleen, and kidney AsA concentrations were observed in these two strains by our analyses. However, a lower concentration of AsA in the adrenal glands of SHR was reported as compared to that of WKY (26). Our results suggest that the effect of the genetic background for hypertension on AsA metabolism in SHR cannot be observed in SHR and WKY because of their ability to synthesize a large amount of AsA. On the other hand, it was demonstrated that SHR-od and ODS rats administered an AsA dose equivalent to the required dose are available models for examining the effect of the genetic background for hypertension on AsA metabolism.

Recently, it has been reported that hypertension causes oxidative stress in some organs (27–30). In our analysis of hepatic TBARS (Fig. 3), indicating the concentration of lipid peroxide, we found that the level of TBARS in SHR-od at 6 wk of age tended to be higher than that in ODS rats, and the level at 28 wk of age was higher. This analysis also demonstrated that hepatic TBARS in SHR-od increased during a 22-wk period, from 6 to 28 wk of age, but did not change in ODS rats. On the other hand, the hepatic activity of GSH-Px, which reduces peroxide using glutathione, was slightly lower in SHR-od than in ODS rats at 6 wk of age. The activity in ODS rats increased during the subsequent 22 wk, but did not change in SHR-od. Thus, the lower activity of hepatic GSH-Px in SHR-od at 6 wk of age might contribute the higher level of hepatic TBARS as compared to ODS rats, and the impairment of the increase in the hepatic activity of GSH-Px during the subsequent 22 wk in SHR-od might cause the elevation of hepatic TBARS. Vérelc et al. also demonstrated a lower activity of hepatic GSH-Px in SHR as compared to WKY (31). Moreover, it has been pointed out by Murakami et al. (32) that erythrocyte GSH-Px activity changed with the development of hypertension and stroke in stroke-prone SHR. This taken together, it is speculated that oxidative stress is caused in the liver of SHR-od, leading to the acceleration of AsA oxidation. It has been reported that the plasma level of AsA is decreased under an oxidatively stressed condition, such as cerebral ischemia (33–35), myocardial infarction (36), and inflammation (4). In further studies, the level of dehydroascorbic acid and subsequent metabolites in various organs including the liver should be compared between SHR-od and ODS rats.

In this study, it was demonstrated for the first time that the tissue AsA concentrations and urinary excretion of AsA in SHR-od are lower than those in ODS rats. This means that the requirement for AsA might be increased to maintain indispensable levels of AsA in the serum and tissues in SHR-od and hypertensive subjects. Further study is needed to confirm that AsA degradation is accelerated in SHR-od as compared to ODS rats.

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REFERENCES


