Intestinal bacterial origin of D-alanine in urine of mutant mice lacking D-amino-acid oxidase

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Urine from mutant mice which lack D-amino-acid oxidase contained a large amount of D-alanine. The alanine content was not changed by changes in the dietary composition or by starvation. However, oral administration of an antibiotic, amoxicillin, decreased the urinary alanine to the normal level. These results suggest that the D-alanine is not of dietary origin but of intestinal bacterial origin.

INTRODUCTION

D-Amino-acid oxidase (EC 1.4.3.3) is an enigmatic enzyme. It catalyses the oxidative deamination of D-amino acids (stereoisomers of naturally occurring L-amino acids) to the corresponding α -oxo acids [1,2]. Almost all higher animals have this enzyme in the kidney, liver and brain [2]. Although its molecular properties and kinetic mechanism have been elucidated in detail [3–8], its physiological role is not known, because D-amino acids are rare in higher animals.

Mutant animals are useful for the study of the functions of enzymes, because examination of physiological changes caused by mutational changes in enzymes often reveal their physiological roles. We have established a mutant mouse strain which lacks D-amino-acid oxidase [9,10]. The mutant ddY/DAO⁻ mice excrete large amounts of methionine and alanine in urine compared with normal mice [11]. These amino acids are mostly D-isomers [12,13]. D-Methionine was found to derive from DL-methionine supplemented in a commercial mouse diet. The ddY/DAO⁻ mice are unable to utilize the absorbed D-methionine due to a lack of D-amino-acid oxidase, so they excrete it in urine.

The source of the D-alanine abundant in the urine of the ddY/DAO⁻ mice is not known, because their diet does not contain DL-alanine, no metabolic pathway from D-methionine to D-alanine is known, and mammals do not have an isomerase which directly converts L-alanine to its D-isomer. Therefore we examined whether the D-alanine was of dietary origin or not. Because bacterial cell walls contain D-alanine as a major constituent [14], we also examined whether the urinary D-alanine came from intestinal bacteria.

MATERIALS AND METHODS

Antibiotics

Cell wall synthesis inhibitors used were amoxicillin (Pasetocin; Kyowa Hakko Kogyo Co., Tokyo, Japan), cefixime (Cefspan; Fujisawa Pharmaceutical Co., Osaka, Japan) and cephalixin (Keflex; Shionogi & Co., Osaka, Japan). Protein synthesis inhibitors used were clindamycin-2-palmitate hydrochloride (Dalacin; Japan Upjohn, Tokyo, Japan), josamycin propionate (Josamy; Yamanouchi Pharmaceutical Co., Tokyo, Japan) and minocycline hydrochloride (Minomycin; Lederle, Tokyo, Japan).

Mice and diet

Normal ddY/DAO⁺ mice and mutant ddY/DAO⁻ mice were raised on a stock diet (Type NMF; Oriental Yeast Co., Tokyo, Japan). This diet contained about 0.04% DL-methionine as a

supplement [12]. Groups of three or four adult male mice were housed in a polycarbonate cage (21.5 cm × 32 cm × 14 cm) with a bedding of wood shavings. They were maintained at 24 ± 2 °C with a 12 h light/dark cycle.

To examine the effects of diet on the urinary levels of alanine and methionine, we fed the ddY/DAO⁻ mice on Rodent Laboratory Chow 5001 (estimated D-methionine content was 0.0037%; Ralston Purina Co., St. Louis, MO, U.S.A.) or a modified NMF diet (Oriental Yeast Co.) in which supplementary DL-methionine in the NMF diet was replaced by an equal amount of L-methionine [12]. The mice were maintained on the new diets for at least 2 weeks before urine collection.

Urine collection and analysis of amino acids in urine

The mice were kept in individual metabolic cages and urine was collected overnight (17:00 h–10:00 h); throughout this time, the mice had free access to food and water.

After urine was passed through deproteinization and cation-exchange chromatography, amino acid concentrations were determined using amino acid analysers (models KLA-5 and 835, Hitachi, Tokyo, Japan) as previously described [11]. Creatinine content in the urine was determined using an assay kit (Wako Pure Chemical Industries, Osaka, Japan). Amino acid concentrations in urine are normalized by its creatinine content. Normalized amino acid concentrations were compared between control mice and experimental groups of mice by Student's *t* test.

Starvation

The ddY/DAO⁻ mice were starved for 24 h (17:00 h–17:00 h), though they were given water *ad libitum*. They were then transferred to individual metabolic cages and urine was collected for the subsequent 17 h (17:00 h–10:00 h), during which time the animals were starved. Because only a small quantity of urine was excreted under these conditions, urine from two or three mice was combined, and amino acid concentrations and creatinine content were measured.

Antibiotic administration

The ddY/DAO⁻ mice were given, *ad libitum*, the stock diet and water containing antibiotics for 7 days. Antibiotics were present in the water either alone or in combination at the following concentrations: amoxicillin, 0.015%; cefixime, 0.005%; clindamycin, 0.012%; minocycline, 0.003%; amoxicillin, 0.01% plus minocycline, 0.002%; and cephalixin, 0.012% plus josamycin, 0.01%. The water (containing antibiotics) and the cages containing the mice were changed every other day. On day 7, the

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mice were transferred to individual metabolic cages and urine was collected overnight, during which time the mice had free access to food and water containing antibiotics. Urinary amino acid concentrations and creatinine content were then measured.

Time course of the decrease in urinary alanine

The time course of the decrease in urinary alanine was examined in the mice given 0.01% amoxicillin plus 0.002% minocycline. Male ddY/DAO⁻ mice were housed for 10 days in individual metabolic cages and were given the stock diet and water containing the antibiotics *ad libitum*. Meanwhile, urine was collected daily (17:00 h–10:00 h) and kept frozen at -20 °C until analysis. The water (containing the antibiotics) and the metabolic cages were changed every day. Urine samples containing 3 µg of creatinine were applied to a silica gel thin-layer plate and developed as previously described [11]. Alanine was detected by using a spray of 0.2% ninhydrin in ethanol on the plate.

RESULTS

Table 1 shows that urine from mutant ddY/DAO⁻ mice lacking D-amino-acid oxidase contained 9.3-fold more alanine and 5.8-fold more methionine than that from normal ddY/DAO⁺ mice. These amino acids were mostly D-isomers [12,13]. These mice were maintained on the Oriental Type NMF stock diet. When the ddY/DAO⁻ mice were given Purina Rodent Laboratory Chow 5001 which contained a low level of supplementary DL-methionine, the amount of urinary methionine decreased to the normal level, but the level of urinary alanine did not change. When they were given the modified NMF diet in which supplementary DL-methionine in the NMF diet was replaced with an equal amount of L-methionine, the amount of urinary methionine also decreased but the concentration of urinary alanine remained high. These results suggest that the methionine in the urine originated from the diet but that the alanine did not. The decreases in urinary methionine observed when the ddY/DAO⁻ mice were fed on the Rodent Laboratory Chow 5001 or the modified NMF diet are consistent with our previous results [12].

When the ddY/DAO⁻ mice were starved, the urinary methionine decreased to the normal level, but the urinary alanine remained at the high level (Table 1). These results are consistent with the above results, i.e. the methionine is of dietary origin whereas the alanine is not.

To test the possibility of an intestinal bacterial origin of the

Table 1. Effects of diet and starvation on the urinary levels of alanine and methionine

Values are means ± s.d. The numbers in parentheses represent the numbers of urine samples examined. Urine from two or three starved mice was combined due to its small quantity, and seven independent urine samples were analysed. **P* < 0.01 compared with the ddY/DAO⁻ mice given the Oriental NMF diet.

Mouse strain	Diet	Urinary amino acid concentration (µmol/mg of creatinine)	
		Alanine	Methionine
ddY/DAO ⁺	Oriental NMF	0.400 ± 0.101 (4)	0.371 ± 0.257 (4)
ddY/DAO ⁻	Oriental NMF	3.708 ± 0.425 (6)	2.143 ± 0.436 (6)
	Purina 5001	3.635 ± 1.190 (5)	0.401 ± 0.164 (5)*
	Modified NMF	3.027 ± 1.156 (5)	0.309 ± 0.174 (5)*
	Starvation	3.451 ± 1.076 (7)	0.571 ± 0.362 (7)*

Table 2. Effects of antibiotics on the urinary levels of alanine and methionine in mutant ddY/DAO⁻ mice lacking D-amino-acid oxidase

Values are means ± s.d. The numbers in parentheses represent the numbers of urine samples examined. **P* < 0.01 compared with the control mice.

Antibiotics	Urinary amino acid concentration (µmol/mg of creatinine)	
	Alanine	Methionine
None	3.708 ± 0.425 (6)	2.143 ± 0.436 (6)
Amoxicillin (0.015%)	0.592 ± 0.147 (5)*	2.439 ± 0.533 (5)
Cefixime (0.005%)	2.659 ± 0.345 (5)*	2.041 ± 0.579 (5)
Clindamycin (0.012%)	3.133 ± 0.793 (5)	2.196 ± 0.314 (5)
Minocycline (0.003%)	3.054 ± 0.612 (4)	1.623 ± 0.134 (4)
Amoxicillin (0.01%) + minocycline (0.002%)	0.475 ± 0.083 (8)*	2.155 ± 0.397 (8)
Cephalexin (0.012%) + josamycin (0.01%)	2.175 ± 0.794 (5)*	1.802 ± 0.820 (5)

Fig. 1. Time course of the decrease in urinary alanine in a mutant mouse given antibiotics

Lane 1, urine from a normal ddY/DAO⁺ mouse without antibiotics. Lanes 2–12, urine collected on days 0–10 respectively from a mutant ddY/DAO⁻ mouse lacking D-amino-acid oxidase continuously given water containing 0.01% amoxicillin plus 0.002% minocycline (starting on day 0). Lane 13, standard alanine (1.5 µg); lane 14, standard methionine (1.5 µg).

urinary D-alanine, we gave water containing antibiotics to the ddY/DAO⁻ mice for 7 days and measured amino acid concentrations in their urine. Table 2 shows that 0.012% clindamycin did not change the urinary level of alanine. Cefixime (0.005%) and a combination of 0.012% cephalexin and 0.01% josamycin lowered the urinary alanine to some extent. However, a combination of 0.01% amoxicillin and 0.002% minocycline lowered the urinary alanine to the normal level. The effective antibiotic of these two was found to be amoxicillin, as amoxicillin alone at a concentration of 0.05% reduced the urinary alanine level, whereas minocycline alone at 0.003% did not. Although urinary alanine was decreased by amoxicillin or amoxicillin plus minocycline, the urinary methionine content was not significantly changed, again confirming the different sources of these amino acids.

The time course of the decrease in the urinary alanine was examined in the ddY/DAO⁻ mice given water containing 0.01% amoxicillin plus 0.002% minocycline. Fig. 1 shows that the alanine content was high in the urine at 1 and 2 days after administration of the antibiotics. However, urinary alanine content decreased on day 3 and was almost the same as that of a normal ddY/DAO⁺ mouse on and after day 5.

These results suggest that the D-alanine abundant in urine from the ddY/DAO⁻ mice comes from intestinal bacteria.

DISCUSSION

D-Alanine, which is abundant in urine from ddY/DAO⁻ mice, is not of dietary origin. It probably comes from intestinal bacteria, as bacterial cell walls contain D-alanine [14], and oral administration of an antibiotic, amoxicillin, lowered urinary alanine in these mice. Our conclusion is consistent with the finding of Hoeprich [15] that serum from mice and guinea pigs reared under conventional conditions contained D-alanine whereas serum from their counterparts grown under germ-free conditions did not. It is very likely that components released from bacteria are absorbed through the intestine of the host animals. In fact, Yang *et al.* [16] showed that rats efficiently digested and utilized *Escherichia coli* when it was force-fed or when it was placed in the caecum. It seems that the mutant ddY/DAO⁻ mice are unable to metabolize the absorbed D-alanine due to a lack of D-amino-acid oxidase, so they excrete it in urine. These results indicate that, in normal mice, D-alanine is constantly metabolized by the oxidase. Metabolism of D-amino acid(s) of bacterial origin would be one of the physiological functions of D-amino-acid oxidase.

It is not known what species of intestinal bacteria are the source of the D-alanine in urine of the ddY/DAO⁻ mice. A large number of species inhabit the alimentary tract of mice [17]. Amoxicillin was effective in decreasing the urinary alanine in the mutant mice. This drug is one of the semi-synthetic penicillins and suppresses both Gram-positive and Gram-negative bacteria [18]. Due to this broad antimicrobial spectrum, the causal bacterial species have not been identified. The effects of amoxicillin on urinary alanine content cannot be due to an overall disturbance of absorption in the intestine, reabsorption in the kidney or metabolism in the whole body, as no change was observed in the urinary excretion of amino acids other than alanine. Furthermore, cessation of antibiotic administration resulted in an increase in alanine excretion (results not shown).

Administration of amoxicillin or amoxicillin plus minocycline for 7 days to the ddY/DAO⁻ mice decreased the urinary alanine. However, prolonged administration of the antibiotics sometimes caused re-accumulation of alanine in urine to the original high level or above (results not shown). This would be related to the appearance of amoxicillin-resistant bacteria in the alimentary tract and/or to the establishment of new microflora after

suppression of normal flora. Alterations in intestinal microflora caused by administration of antibiotics have been described [19].

We thank Ms. C. Yamanaka for the amino acid analysis of some urine samples. We also thank Ms. Anna Schatz for advice in preparing this manuscript.

REFERENCES

1. Krebs, H. A. (1935) *Biochem. J.* **29**, 1620–1644
2. Meister, A. (1965) *Biochemistry of the Amino Acids*, 2nd edn., vol. 1, pp. 297–304, Academic Press, New York
3. Bright, H. J. & Porter, D. J. T. (1975) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn., vol. 12, Part B, pp. 421–505, Academic Press, New York
4. Yagi, K. (1980) in *New Horizons in Biological Chemistry* (Koike, M., Nagatsu, T., Okuda, J. & Ozawa, T., eds.), pp. 257–270, Japan Scientific Societies Press, Tokyo
5. Ronchi, S., Minchiotti, L., Galliano, M., Curti, B., Swenson, R. P., Williams, C. H., Jr. & Massey, V. (1982) *J. Biol. Chem.* **257**, 8824–8834
6. Fukui, K., Watanabe, F., Shibata, T. & Miyake, Y. (1987) *Biochemistry* **26**, 3612–3618
7. Miura, R. & Miyake, Y. (1988) *Bioorg. Chem.* **16**, 97–110
8. Momoi, K., Fukui, K., Watanabe, F. & Miyake, Y. (1988) *FEBS Lett.* **238**, 180–184
9. Konno, R. & Yasumura, Y. (1983) *Genetics* **103**, 277–285
10. Konno, R. & Yasumura, Y. (1984) *J. Neurochem.* **42**, 584–586
11. Konno, R., Isobe, K., Niwa, A. & Yasumura, Y. (1988) *Biochim. Biophys. Acta* **967**, 382–390
12. Konno, R., Isobe, K., Niwa, A. & Yasumura, Y. (1988) *Metab. Clin. Exp.* **37**, 1139–1142
13. Konno, R., Nagata, Y., Niwa, A. & Yasumura, Y. (1989) *Biochem. J.* **261**, 285–287
14. Wheat, R. W. (1988) in *Zinsser Microbiology* (Joklik, W. K., Willett, H. P., Amos, D. B. & Wilfert, C. M., eds.), 19th edn., pp. 63–76, Appleton & Lange, Norwalk, CT
15. Hoeprich, P. D. (1965) *J. Biol. Chem.* **240**, 1654–1660
16. Yang, M. G., Bergen, W. G., Sculthorpe, A. E. & Mickelsen, O. (1972) *Proc. Soc. Exp. Biol. Med.* **139**, 1312–1316
17. Mitsuoka, T. (1986) *Chonai Kinsou no Bunrui to Seitai* (Classification and Ecology of Intestinal Microflora), Shokuseikatsu Kenkyu Kai, Tokyo (in Japanese)
18. Willett, H. P. (1988) in *Zinsser Microbiology* (Joklik, W. K., Willett, H. P., Amos, D. B. & Wilfert, C. M., eds.), 19th edn., pp. 128–160, Appleton & Lange, Norwalk, CT
19. Jawetz, E., Melnick, J. L., Adelberg, E. A., Brooks, G. F., Butel, J. S. & Ornston, L. N. (1989) in *Medical Microbiology*, 18th edn., p. 151, Appleton & Lange, Norwalk, CT

Received 28 November 1989/29 January 1990; accepted 8 February 1990