Involvement of D-amino acid oxidase in elimination of free D-amino acids in mice

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The physiological role of D-amino acid oxidase was investigated by using mutant ddY/DAO⁻ mice lacking the enzyme. Free D-amino acid concentrations in the mutant mice were significantly higher than those of control ddY/DAO⁺ mice in kidney, liver, lung, heart, brain, erythrocytes, serum and urine. The results suggest that the enzyme is involved in the catabolism of free D-amino acids in the body, and that free D-amino acids are also excreted into urine.

INTRODUCTION

D-Amino acid oxidase (DAO, EC 1.4.3.3) is a flavo-enzyme widespread in many animal tissues such as kidney, liver, brain, leucocytes and heart (Meister, 1965). The enzyme catalyses the oxidative deamination of neutral free D-amino acids to produce α -oxo acids:

The substrates of the enzyme *in vitro* are solely D-isomers of free neutral amino acids such as alanine, methionine, proline, tyrosine, isoleucine, leucine, phenylalanine, serine, valine and tryptophan (Meister & Wellner, 1963).

In spite of its well-studied enzymic and physico-chemical properties, the physiological function of the enzyme remains unknown. Hamilton *et al.* (1979) suggested that the physiological substrate of DAO is a product of reaction of cysteamine with glyoxylate. According to their experiments *in vitro*, the product reacted well with DAO, and Fitzpatrick & Massey (1982) reported that the catalytic-centre activity obtained with the substrate was similar to that with D-proline. Another basis for the proposal was that the amount of DAO present in various mammalian tissues seemed too great in relation to free D-amino acids. In fact, it is generally believed that mammals neither synthesize nor possess D-amino acids (Hoeprich, 1965). However, we have recently found substantial amounts of free D-amino acids in plasma from patients with renal diseases (Nagata *et al.*, 1987). Since DAO is present at the highest activity in the kidney compared with any other organ (Burch *et al.*, 1958), the finding led us to suspect that free D-amino acids are the substrates for DAO *in vivo*. In order to clarify this point, a DAO-lacking mutant mouse strain was used to compare the amounts of neutral free D-amino acids in various tissues with those in normal control animals.

MATERIALS AND METHODS

The mutant mouse strain ddY/DAO⁻, lacking DAO activity, was established as described previously (Konno & Yasumura, 1983, 1984a) and raised on a stock diet (type NMF; Oriental Yeast, Tokyo, Japan) together

with the normal control ddY/DAO⁺ mice. Male mutant and normal mice (11 weeks old) were chosen for the experiment, since male mice possess a higher DAO activity than females (Konno & Yasumura, 1983). In order to prevent the oral ingestion of free D-amino acids, the mice were kept for 2 weeks before the experiment on a diet which did not include D-amino acids as supplement (Type NIH-improved; Oriental Yeast) and on mesh to prevent access to bacterially contaminated faeces. The mice were starved, but allowed water, for 16–19 h before being killed, by bleeding from axillary vessels under anaesthesia with diethyl ether. After rinsing with phosphate-buffered saline [150 mM-NaCl/10 mM-sodium phosphate buffer (pH 7.4)] to remove blood, the whole organs of liver, kidney, brain, heart and lung were stored for not more than 30 days at –80 °C until use.

The organ was minced into small pieces, homogenized with 4–5 vol. of the phosphate-buffered saline in a glass homogenizer in an ice bucket, at 1000 rev./min for 1 min, and centrifuged at 160 000 *g* for 15 min at 4 °C. The erythrocytes were homogenized in a similar fashion with 5 vol. of distilled water, and centrifuged at 16 500 *g* for 10 min at 4 °C. The supernatant extracts were passed through a 'Centricut' ultra-membrane filter (Kurabo, Osaka, Japan), which removes substances larger than 10 000 Da, by centrifuging at 2000 *g* for 30 min at 4 °C. The filtrates were used in the assay for D-amino acids after storage at –30 °C for not longer than 7 days.

Free D-amino acids were assayed as described elsewhere (Nagata *et al.*, 1985). Free D-amino acids serve as substrates for DAO to produce α -oxo acids, which were detected spectrophotometrically at 445 nm as hydrazone derivatives. The assay method for D-amino acids also weakly detects glycine and L-proline (Meister & Wellner, 1963). However, the rate of colour formation due to glycine was 1/200 of that due to D-alanine, and that due to L-proline was far less than that with glycine in our assay system (results not shown). Glycine does not seem to have been included in our measurements of D-amino acids, because an amino acid analysis (Hitachi Amino Acid Analyser L8500) of samples obtained from kidney extracts showed no difference in the free glycine content between the mutant and the control mice, and almost no free proline was detected by the analysis (cf. Table 1). No

Table 1. Free D-amino acid content of normal and DAO-lacking mutant-mouse tissues

Neutral free D-amino acids of various tissue extracts from ddY/DAO⁺ normal and ddY/DAO⁻ mutant mice were measured as described in the Materials and methods section. The values are means $\pm$ S.E.M. for the numbers of observations shown in parentheses. Data were subjected to Welch's test (Trickett *et al.*, 1956) to assess the significance of any difference between the two groups.

Mice ...	Free D-amino acid content (nmol/g wet wt.)		
	ddY/DAO ⁺	ddY/DAO ⁻	
Kidney	97.4 $\pm$ 12.0 (5)	562.4 $\pm$ 63.8 (7)	<i>P</i> < 0.0005
Liver	94.3 $\pm$ 10.5 (5)	178.3 $\pm$ 17.2 (7)	<i>P</i> < 0.005
Lung	97.5 $\pm$ 23.6 (5)	194.8 $\pm$ 23.6 (7)	<i>P</i> < 0.01
Heart	38.1 $\pm$ 15.9 (5)	131.0 $\pm$ 12.7 (7)	<i>P</i> < 0.005
Brain	32.7 $\pm$ 9.7 (5)	96.9 $\pm$ 10.7 (7)	<i>P</i> < 0.005
Serum (nmol/ml)	11.1 $\pm$ 4.4 (5)	57.7 $\pm$ 3.5 (7)	<i>P</i> < 0.0001
Erythrocytes (nmol/ml of blood)	60.2 $\pm$ 13.5 (5)	257.5 $\pm$ 48.1 (7)	<i>P</i> < 0.01
Urine (nmol/mg of creatinine)	301 $\pm$ 81 (5)	4030 $\pm$ 670 (6)	<i>P</i> < 0.005

enzyme has been found in mammals that can hydrolyse peptides containing D-amino acid residues, nor racemases that might produce D-amino acids from their L-isomers. Hence it is suggested that solely D-isomers of free neutral amino acids which existed in the tissues, serum or urine were measured by the assay procedure used in the present experiments. Urinary creatinine was determined with a kit (Serotec, Sapporo, Japan), in order to express the D-amino acid content per mg of creatinine in the urine.

DAO from pig kidney was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were analytical-grade products.

RESULTS AND DISCUSSION

Neutral free D-amino acids were measured in mutant (ddH/DAO⁻) mice lacking DAO, as well as in the normal control (ddY/DAO⁺) mice having the enzyme. In all tissues of the mutant mice, free D-amino acid concentrations were significantly higher than in control mice (Table 1). In particular, the concentrations of D-amino acids in the kidney and serum of the mutant were respectively 6- and 5-fold higher than in the controls. The concentration of free D-amino acids in the urine of the mutant was 13 times that of the control. Urinary excretion is estimated approximately for the mutant group as 2860 nmol/day, assuming a daily production of urine of 2 ml and a urinary creatinine concentration of 0.34 mg/ml.

In spite of the increased excretion of D-amino acids into the urine, a remarkably higher concentration of D-amino acids was still observed in the various tissues of mutant mice. This suggests that, in the normal mice, the increased amounts of D-amino acids shown in the mutant are being catabolized by DAO, and that DAO is involved in the elimination of D-amino acids in the mice. Thus a view that the role of DAO is, in part, to catalyse the oxidative deamination of free D-amino acids is feasible. This view is strengthened by the observation that the tissue distribution pattern of free D-amino acids (Table 1) is similar to that of DAO (Burch *et al.*, 1958; Horiike *et*

al., 1985). In this regard we have recently found DAO also in mouse liver (Nagata & Akino, 1988; Nagata *et al.*, 1988), which was previously assumed to be devoid of the enzyme. Further support comes from observations that DAO contributes to the utilization of certain D-amino acids fed as nutrients, in renal epithelial cells in culture (Gilbert & Migeon, 1975) and in mice *in vivo* (Konno & Yasumura, 1984b), and that DAO⁻ mutant mice excrete much more D-methionine present in the diet than do normal control mice (Konno *et al.*, 1988).

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