Role of renal D-amino-acid oxidase in pharmacokinetics of D-leucine

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Hasegawa, Hiroshi, Takehisa Matsukawa, Yoshihiko Shinohara, Ryuichi Konno, and Takao Hashimoto. Role of renal D-amino-acid oxidase (DAO) in rats suggested that the elimination behavior of D-[2H7]leucine observed in rats was slowed down the elimination of D-amino acids, because the α-keto acid and L-amino acid formed are indistinguishable from endogenous compounds. First, we examined whether DAO is indispensable for conversion of D-amino acids to their α-keto acids by using the stable isotope tracer technique. After a bolus intravenous administration of D-[2H7]leucine to mutant mice lacking DAO activity (ddY/DAO+/H11002), elimination of D-[2H7]leucine and formation of α-[2H7]ketoisocaproic acid ([2H7]KIC) and L-[2H7]leucine in plasma were determined. The ddY/DAO− mice, in contrast to ddY/DAO+ mice, failed to convert D-[2H7]leucine to [2H7]KIC and L-[2H7]leucine. This result clearly revealed that DAO was indispensable for the process of chiral inversion of D-leucine. We further investigated the effect of renal mass reduction by partial nephrectomy on elimination of D-[2H7]leucine and formation of [2H7]KIC and L-[2H7]leucine. Renal mass reduction slowed down the elimination of D-[2H7]leucine. The fraction of conversion of D-[2H7]leucine to [2H7]KIC in sham-operated rats was 0.77, whereas that in five-sixths-nephrectomized rats was 0.25. The elimination behavior of D-[2H7]leucine observed in rats suggested that kidney was the principal organ responsible for converting D-leucine to KIC.

α-ketoisocaproic acid; kidney; nephrectomy

ALL AMINO ACIDS, EXCEPT FOR GLYCINE, OCCUR AS OPTICALLY ACTIVE ISOMERS. AMINO ACIDS USED IN PROTEIN SYNTHESIS ARE OF THE L-CONFIGURATION, WHEREAS D-AMINO ACIDS RARELY OCCUR IN PROTEINS. RECENT PROGRESS IN CHROMATOGRAPHY ON THE SEPARATION OF D-,L-AMINO ACIDS REVEALS THAT SIGNIFICANT AMOUNTS OF SEVERAL FREE D-AMINO ACIDS ARE PRESENT IN HIGHER ANIMALS (5). A NUMBER OF STUDIES HAVE INDICATED THAT D-AMINO ACIDS PLAY ESSENTIAL ROLES IN SEVERAL PHYSIOLOGICAL FUNCTIONS (2, 9, 17). BECAUSE D-AMINO ACIDS HAVE ALSO BEEN FOUND IN FOODS AND BEVERAGE DRINKS (3), IT IS IMPORTANT TO UNDERSTAND HOW THEIR LEVELS ARE CONTROLLED.

D-Amino-acid oxidase (DAO; EC 1.4.3.3) is a flavoenzyme that catalyzes the oxidation of D-amino acids to the corresponding α-keto acids (15). These α-keto acids are stereospecifically converted to their corresponding L-amino acids by transaminases. Almost all higher animals have DAO in their kidney, liver, and brain, although the mouse is an exception and does not have the enzyme in the liver (11). Because DAO is present at the highest activity in the kidney compared with the other organs, injury to the kidney may cause accumulation of D-amino acids. Increasing evidence that plasma levels of D-amino acids were significantly higher in patients with renal diseases than in healthy subjects (1, 18) suggests that renal DAO plays a prominent role in elimination of D-amino acids.

One of the unique advantages of the use of a stable isotope-labeled compound as a tracer is that an endogenous compound and its exogenously administered labeled analog are separately measurable by using gas chromatography-mass spectrometry (GC-MS). Our recent use of stable isotope-labeled D-leucine (D-[2H7]leucine) has proved a powerful methodology for examining the pharmacokinetic behavior of exogenously administered D-leucine and studying the conversion of D-leucine to the corresponding α-keto acid, α-ketoisocaproic acid (KIC), and L-leucine (6–8, 16). It became apparent that ~30% of an administered dose of D-[2H7]leucine in rats was converted to the L-enantiomer through [2H7]KIC as an intermediate.

The purpose of this study was to explore the hypothesis that D-leucine is predominantly metabolized by renal DAO to KIC. To confirm the role of DAO, we investigated by using the stable isotope tracer technique whether mutant mice lacking DAO activity (ddY/DAO−) are able to convert D-leucine to KIC and/or L-leucine. We further investigated the pharmacokinetics of exogenously administered D-leucine in five-sixths-nephrectomized rats to establish the role of renal DAO.

MATERIALS AND METHODS

**Chemicals.** Optically pure D- and L-[4,5,5,6,6-2H5]leucine (D-[2H5]leucine and L-[2H5]leucine, respectively; >98 atom % 2H and >99.8% e.e., each) and sodium [4,5,5,6,6-2H5]2-oxo-4-methylpentanoate ([2H5]KIC Na, >98 atom % 2H) were prepared from [2H5]leucine in our laboratory as described previously (7, 16). D-[2,3,3-2H3]leucine and sodium [5,5,5-2H3]2-oxo-4-methylpentanoate ([2H3]KIC Na) were purchased from Isotec (Miamisburg, OH). (S)-(+)-(+)α-methoxy-α-trifluoromethyl-phenylacetyl chloride ([+-]MTPA-Cl, >99% α-[4,5,5,6,6,6-2H6]ketoisocaproate) and 10% HCl in methanol were purchased from Tokyo Kasei (Tokyo, Japan). N-phenyl-phenylene-1,2-diamine was purchased from Aldrich (Milwaukee, WI).

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Animals. The experimental protocols were approved by the Institutional Animal Care Committee of Tokyo University of Pharmacy and Life Science. Mutant ddY/DAO− mice lacking DAO activity were described before (10). Male ddY/DAO− mice and normal ddY/DAO+ mice (7 wk old) were chosen for the experiment. Male Sprague-Dawley rats (7 wk old) were obtained from Tokyo Laboratory Animal Center (Tokyo, Japan). These animals were maintained in an air-conditioned room at 23 ± 1°C and 55 ± 5% humidity on a 12:12-h dark-light cycle. All animals were allowed free access to water and food (CE-2; Clea Japan, Tokyo, Japan).

Surgery. Rats (n = 6) were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), and the right kidneys were removed. At 3 days after the first surgery, the anterior and posterior apical segmental branches of the left kidney were removed under anesthesia. Rats (n = 6) underwent sham operations with decapsulation of both kidneys at the same intervals as nephrectomy. After surgery, animals were allowed free access to water and food for 3 days.

Dose experiments. The ddY/DAO− and ddY/DAO+ mice (n = 6 each) were fasted overnight. D-[2H7]-leucine was dissolved in saline (70 μmol/ml) and administered (35 μmol/kg body wt) into the tail vein. The mice were killed by cervical dislocation at 5 or 60 min after the administration, and a blood sample (300 μl) was collected from the inferior vena cava. The blood was centrifuged to separate plasma at 3,000 rpm for 15 min. The plasma was stored at −20°C until analysis.

After an overnight fast, five-sixths (5/6)-nephrectomized and sham-operated rats (n = 6 each) were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). D-[2H7]-leucine (35 μmol/kg body wt) was administered into the femoral vein. Blood samples (150 μl) were obtained from the jugular vein 10 min before and 0.5, 1, 3, 5, 10, 15, 20, 30, 60, 90, 120, 180, 240, 300, and 360 min after the administration. The blood was centrifuged to separate plasma at 3,000 rpm for 10 min. The plasma was stored at −20°C until analysis.

Sample preparation for GC-MS-selected ion monitoring. D-[2H7]-leucine (1 nmol) and [2H7]KIC (0.1 nmol) were added to plasma samples (50 μl each) as analytic internal standards for the measurement of D-[2H7]-leucine, L-[2H7]-leucine, [2H7]-KIC, D-leucine, L-leucine, and KIC. All the plasma samples were then subjected to GC-MS-selected ion monitoring (SIM) analysis, and the plasma concentrations of D-[2H7]-leucine, L-[2H7]-leucine, [2H7]-KIC, D-leucine, L-leucine, and KIC were determined by the isotope dilution method as described previously (8).

GC-MS-SIM. The GC-MS-SIM analysis was conducted on a Shimadzu (Kyoto, Japan) QP1000EX gas chromatograph-mass spectrometer equipped with a data processing system. The operating conditions were the same as those described in previous publications (6, 16).

For leucine enantiomer analysis, the GC-MS conditions were as follows. The initial column temperature was set at 120°C. After the sample injection, temperature was maintained for 2 min, increased at 15°C/min to 190°C, and held at 190°C for 3 min. The mass spectrometer was operated in chemical ionization mode at 70 eV in electron impact ionization mode.

For KIC analysis, the GC-MS conditions were as follows. The initial column temperature was set at 120°C. After the sample injection, temperature was maintained for 2 min, increased at 20°C/min to 190°C, and held at 190°C for 3 min. The mass spectrometer was operated in impact ionization mode at 70 eV. SIM was performed on the molecular acid.
Fig. 4. Plasma concentrations of d-[2H₇]leucine, [2H₇]KIC, and l-[2H₇]leucine at 5 and 60 min after administration of d-[2H₇]leucine (35 μmol/kg body wt iv) to normal ddY/DAO⁺ and mutant ddY/DAO⁻ mice. Values represent mean ± SD (n = 6). *Not detected; **P < 0.0005 compared with normal mice.

Fig. 5. Plasma concentrations of endogenous d-leucine, KIC, and l-leucine at 5 and 60 min after administration of d-[2H₇]leucine (35 μmol/kg body wt iv) to normal ddY/DAO⁺ and mutant ddY/DAO⁻ mice. Values represent means ± SD (n = 6). No statistical differences were found between ddY/DAO⁺ and ddY/DAO⁻ mice except for d-leucine in the plasma. *Not detected.

RESULTS

Metabolism of d-[2H₇]leucine in mutant mice lacking DAO activity. After a bolus intravenous administration of d-[2H₇]leucine to mutant ddY/DAO⁻ mice and normal ddY/DAO⁺ mice, the plasma concentrations of the labeled and nonlabeled leucine enantiomers and KIC were determined by GC-MS-SIM (Figs. 2 and 3). As shown in Fig. 4, the plasma levels of d-[2H₇]leucine at 5 min after the administration were similar in ddY/DAO⁺ mice (49.4 ± 15.5 nmol/ml) and ddY/DAO⁻ mice (60.8 ± 5.1 nmol/ml). However, a significant difference in those at 60 min was observed (32.8 ± 5.7 vs. 53.8 ± 3.4 nmol/ml, P < 0.0005). Formations of [2H₇]KIC and L-[2H₇]leucine were observed in ddY/DAO⁺ mice, whereas no
detectable amounts of \([^{2}H_7]\)KIC and l-[\(^{2}H_7\)]leucine were present in the plasma from ddY/DAO\(^{-}\) mice (Figs. 2–4).

No detectable amounts of endogenous d-leucine were observed in the plasma from ddY/DAO\(^{+}\) mice, whereas considerable amounts of endogenous d-leucine were found in ddY/DAO\(^{-}\) mice, which were ~5% of total endogenous leucine (Fig. 5). On the other hand, there were no differences in endogenous KIC and l-leucine concentrations between ddY/DAO\(^{-}\) and ddY/DAO\(^{+}\) mice (Fig. 5).

Effect of renal mass reduction on pharmacokinetics of d-[\(^{2}H_7\)]leucine in rats. After a bolus intravenous administration of d-[\(^{2}H_7\)]leucine to sham-operated and 5/6-nephrectomized rats, plasma concentration of d-[\(^{2}H_7\)]leucine, [\(^{2}H_7\)]KIC, and l-[\(^{2}H_7\)]leucine were examined at various times over 6 h (Fig. 6). Endogenous leucine enantiomers and KIC were measured simultaneously. d-[\(^{2}H_7\)]leucine disappeared biexponentially in both rats (Fig. 6A), but the systemic plasma clearance was significantly lower in 5/6-nephrectomized rats than in sham-operated rats (1.2 ± 0.3 vs. 6.4 ± 2.1 ml·min\(^{-1}\)·kg\(^{-1}\), \(P < 0.005\); Table 1).

The appearance of [\(^{2}H_7\)]KIC into the plasma was very rapid (Fig. 6B). In sham-operated rats, the plasma concentration of [\(^{2}H_7\)]KIC had already reached maximum concentration (2.7 ± 0.6 nmol/ml) in the first plasma sample taken 0.5 min after administration of d-[\(^{2}H_7\)]leucine. In 5/6-nephrectomized rats, the plasma concentration of [\(^{2}H_7\)]KIC reached maximum (0.3 ± 0.2 nmol/ml) at 1 min after the administration. The AUC\(_{0-360 \text{ min}}\) values of [\(^{2}H_7\)]KIC in 5/6-nephrectomized rats were markedly decreased to 28% compared with those in sham-operated rats (24 ± 12 vs. 86 ± 18 min·nmol·ml\(^{-1}\), \(P < 0.0005\)).

The appearance of l-[\(^{2}H_7\)]leucine into the plasma was also rapid in both rats (Fig. 6C). In the plasma at 1 min after administration of d-[\(^{2}H_7\)]leucine, the concentration of l-[\(^{2}H_7\)]leucine reached maximum (5/6 nephrectomized, 0.9 ± 0.8 nmol/ml vs. sham operated 4.7 ± 1.7 nmol/ml). The AUC\(_{0-360 \text{ min}}\) values of l-[\(^{2}H_7\)]leucine in 5/6-nephrectomized rats were significantly decreased to 22% compared with those in sham-operated rats (123 ± 45 vs. 551 ± 182 min·nmol·ml\(^{-1}\), \(P < 0.005\)).

In both groups of rats, the plasma concentration of endogenous l-leucine was almost constant for 6 h after administration of d-[\(^{2}H_7\)]leucine (Fig. 7A), and no detectable amounts of endogenous d-leucine were found. As shown in Fig. 7B, the plasma concentration of endogenous KIC in 5/6-nephrectomized rats was almost constant for 6 h. On the other hand, KIC in sham-operated rats tended to fall gradually, reaching the minimum value of 16.8 ± 5.3 nmol/ml at 15 min after the administration, and then increased steadily, which was consistent with our previous study in normal rats (8).

**DISCUSSION**

Mutant mice lacking DAO activity were found in the ddY strain and had been established as inbred strains through brother-sister mating (10, 12, 14). The mutant ddY/DAO\(^{-}\) mice have a missense mutation (G541A) in the DAO gene, which causes the loss of enzyme activity (20). The ddY/DAO\(^{-}\) mice, in contrast to normal ddY/DAO\(^{+}\) mice, could not utilize d-phenylalanine for growth in place of its l-isomer. However, both ddY/DAO\(^{-}\) and ddY/DAO\(^{+}\) mice utilized phenylpyruvic

![Fig. 6. Plasma concentration time profiles for d-[\(^{2}H_7\)]leucine (A), [\(^{2}H_7\)]KIC (B), and l-[\(^{2}H_7\)]leucine (C) in sham-operated and 5/6-nephrectomized (5/6-Nx) rats after administration of d-[\(^{2}H_7\)]leucine (35 \(\mu\)mol/kg body wt iv). Values represent mean ± SD (\(n = 6\)). Simulated curves obtained from calculated rate constants are represented by the lines in (A) and (B).](http://ajpendo.physiology.org/)

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Table 1. Pharmacokinetic parameters for \( d-[\text{H}_7] \)leucine, \( [\text{H}_7] \)KIC, and \( l-[\text{H}_7] \)leucine in sham-operated and 5/6-nephrectomized rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>5/6-Nephrectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>( d-[\text{H}_7] )leucine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( t_{1/2}, \text{min} )</td>
<td>124 ( \pm ) 20</td>
<td>447 ( \pm ) 139*</td>
</tr>
<tr>
<td>( \text{AUC}_{0-\infty}, \text{min} \cdot \text{nmol} \cdot \text{ml}^{-1} )</td>
<td>6.603 ( \pm ) 1.835</td>
<td>30.558 ( \pm ) 6.489†</td>
</tr>
<tr>
<td>CL, ml/min \cdot kg(^{-1} )</td>
<td>6.4 ( \pm ) 2.1</td>
<td>1.2 ( \pm ) 0.3*</td>
</tr>
<tr>
<td>( [\text{H}_7] )KIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{AUC}_{0-360 \text{min}}, \text{min} \cdot \text{nmol} \cdot \text{ml}^{-1} )</td>
<td>86 ( \pm ) 18</td>
<td>24 ( \pm ) 12†</td>
</tr>
<tr>
<td>( l-[\text{H}_7] )leucine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{AUC}_{0-360 \text{min}}, \text{min} \cdot \text{nmol} \cdot \text{ml}^{-1} )</td>
<td>551 ( \pm ) 182</td>
<td>123 ( \pm ) 45*</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SD. AUC, area under the curve; KIC, \( \alpha \)-ketoisocaproic acid; CL, clearance. *P < 0.005; †P < 0.0005.

The present study directly shows that ddY/DAO\(^{-} \) mice, in contrast to ddY/DAO\(^{+} \) mice, failed to convert \( d-[\text{H}_7] \)leucine to \( [\text{H}_7] \)KIC and \( l-[\text{H}_7] \)leucine. Because ddY/DAO\(^{-} \) mice are considered to have the ability to convert KIC to \( l \)-leucine, their conversion failure is due to the defect of DAO activity. This result clearly revealed that DAO was dispensable for the process of chiral inversion of \( \delta \)-leucine and that the sequential conversion through KIC as an intermediate was the only pathway for conversion of \( \delta \)-leucine to the \( \text{L} \)-enantiomer.

Considerable amounts of endogenous \( \delta \)-leucine were detected in the plasma of ddY/DAO\(^{-} \) mice, which was consistent with the report by Hamase et al. (4). We have determined the amounts of \( \delta \)-leucine in the diet (CE-2) by use of GC-MS-SIM and found that a considerable amount of \( \delta \)-leucine (20.0 nmol/g) is present. To our knowledge, there is no literature that demonstrates the presence of the biosynthesis of \( \delta \)-leucine in higher animals. Thus dietary \( \delta \)-leucine might be one of the possible sources.

In the present study, we found that renal mass reduction by partial nephrectomy slowed down the elimination of \( d-[\text{H}_7] \)leucine in rats. Systemic plasma clearance of \( d-[\text{H}_7] \)leucine in 5/6-nephrectomized rats was one-sixth of that in sham-operated rats. It should be noted that the decrease in plasma clearance of \( d-[\text{H}_7] \)leucine in 5/6-nephrectomized rat might be due to decreased elimination of \( d-[\text{H}_7] \)leucine other than the conversion of \( d-[\text{H}_7] \)leucine to \( [\text{H}_7] \)KIC, such as in urinary excretion. In an attempt to distinguish between the conversion and other elimination, rate constants were calculated on the basis of the kinetic model shown in Fig. 1. In sham-operated rats, the rate constants for the conversion of \( d-[\text{H}_7] \)leucine to \( [\text{H}_7] \)KIC (\( k_{31} \)) and the elimination of \( d-[\text{H}_7] \)leucine other than the conversion of \( d-[\text{H}_7] \)leucine to \( [\text{H}_7] \)KIC (\( k_{01} \)) were 0.02 and 0.006 min\(^{-1} \), respectively (Table 2). On the other hand, \( k_{31} \) in 5/6-nephrectomized rats (0.0016 min\(^{-1} \)) was one-twelfth of that in sham-operated rats, whereas \( k_{01} \) in 5/6-nephrectomized rats (0.0049 min\(^{-1} \)) was four-fiftieths of that in sham-operated rats. These results showed that the renal mass reduction mainly caused a decrease of the conversion of \( d-[\text{H}_7] \)leucine to \( [\text{H}_7] \)KIC.

The fraction of conversion of \( d-[\text{H}_7] \)leucine to \( [\text{H}_7] \)KIC was obtained by the ratio between the total elimination rate constants of \( d-[\text{H}_7] \)leucine and the rate constant for the conversion, that is, \( k_{31}/(k_{01} + k_{31}) \). The value in sham-operated rats was estimated to be 0.77, which was almost consistent with the value in our previous study in normal rats (8). On the other hand, the fraction in 5/6-nephrectomized rats was markedly decreased to 0.25 (32%), which resulted from the DAO activity in the remnant kidney and other organs such as liver and brain.

Table 2. Rate constants for the kinetic analysis model on intravenous administration of \( d-[\text{H}_7] \)leucine to sham-operated and 5/6-nephrectomized rats

<table>
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<tr>
<th>Rate Constant, min(^{-1} )</th>
<th>Sham</th>
<th>5/6-Nephrectomized</th>
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<tr>
<td>( k_{0} )</td>
<td>0.006</td>
<td>0.0049</td>
</tr>
<tr>
<td>( k_{21} )</td>
<td>0.215</td>
<td>0.288</td>
</tr>
<tr>
<td>( k_{12} )</td>
<td>0.051</td>
<td>0.087</td>
</tr>
<tr>
<td>( k_{31} )</td>
<td>0.020</td>
<td>0.0016</td>
</tr>
<tr>
<td>( k_{01} )</td>
<td>1.334</td>
<td>0.617</td>
</tr>
</tbody>
</table>

For explanation of rate constants, see MATERIALS AND METHODS.
The decrease of renal DAO contents by nephrectomy may compensate DAO activity by inducing DAO. Nagata et al. (19) observed that DAO was not inducible in adult mice even with a high dose of D-alanine. Thus, assuming that DAO in liver and brain was not induced by nephrectomy, the DAO activity in kidney (x) and that in the other organs (y) were estimated by the following two simultaneous equations: x + y = 0.77 and x/6 + y = 0.25, and were calculated to be 0.62 and 0.15, respectively. Namely, the ratio of conversion in kidney occupied ~80% of overall conversion. These results suggested that the kidney was the principal organ responsible for converting D-leucine to KIC in vivo.

In conclusion, the present study shows that renal DAO plays the principal role in the metabolism of D-leucine in vivo. Because urinary excretion may also play a role in the elimination of D-leucine, it is important to establish the quantitative roles of metabolism by DAO compared with urinary excretion in both intact and 5/6-nephrectomized rats. We are now in the process of extending this approach to elucidate the overall role of the kidney in the elimination of D-leucine.

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