Brief Report

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Evaluation of D-amino acid oxidase activity in rat kidney using a D-kynurenine derivative, 6-methylthio-D-kynurenine: An *in vivo* microdialysis study

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SUMMARY D-Amino acid oxidase (DAO), a D-amino acid metabolizing enzyme, is reportedly associated with the psychiatric disease schizophrenia, suggesting a role for DAO inhibitors in its treatment. We have previously reported that DAO catalyzes the conversion of nonfluorescent 6-methylthio-D-kynurenine (MeS-D-KYN) to fluorescent 5-methylthiokynurenic acid (MeS-KYNA) *in vitro*. The present study aimed to determine the potential of MeS-D-KYN in evaluating DAO activity *in vivo* using renal microdialysis technique in rats. Male Sprague-Dawley rats were subjected to linear microdialysis probe implantation in the left kidney. Continuous perfusion of MeS-D-KYN was maintained, and DAO activity in the kidney cortex was evaluated by measuring the MeS-KYNA content in the microdialysate. The microdialysate was collected every 30 min and analyzed by high-performance liquid chromatography with fluorescence detection, monitored at 450 nm with an excitation wavelength of 364 nm. A significant production of MeS-KYNA was observed during, but not before, infusion of MeS-D-KYN, indicating that this compound is not endogenous. MeS-KYNA production was suppressed by the co-infusion of DAO inhibitor, 5-chlorobenzo[*d*]isoxazol-3-ol (CBIO), suggesting that MeS-D-KYN was converted to MeS-KYNA by renal DAO. Moreover, oral administration of CBIO effectively suppressed DAO activity in a dose-dependent manner. DAO converted MeS-D-KYN to MeS-KYNA *in vivo*, suggesting the potential of this compound in evaluating DAO activity. The use of the renal microdialysis technique developed in this study facilitates the monitoring of DAO activity in live experimental animals.

Keywords 6-methylthio-D-kynurenine, microdialysis, D-amino acid oxidase, rat kidney

1. Introduction

D-Amino acid oxidase (DAO; EC.1.4.3.3), an enzyme that metabolizes endogenous D-amino acids, especially D-serine, has been reported to be associated with schizophrenia (*1-3*). DAO activity was reportedly increased in the postmortem cortex of patients with schizophrenia (*3*), suggesting that identification of a DAO activity inhibitor might lead to the development of novel therapeutics for schizophrenia (*4*).

The general, conventional assay for the evaluation of DAO activity requires a two-step reaction system consisting of an enzyme reaction with DAO, followed by a color development reaction of co-produced H_2O_2 with peroxidase (POD) and some colorants. However, the inhibitory activity cannot be assessed if a test compound can inhibit POD or is self-oxidized by POD. As POD and chromophore or fluorogenic substances are used in the conventional DAO assay, this technique has some inherent disadvantages in the screening of DAO inhibitors.

Recently, we designed and developed 6-methylthio-D-kynurenine (MeS-D-KYN) as a fluorescence probe for evaluating DAO activity (*5*). MeS-D-KYN is a non-fluorescent compound that is converted to a fluorescent compound, 5-methylthiokynurenic acid (MeS-KYNA), by DAO (Scheme 1). We observed blue fluorescence of MeS-KYNA after an *in vitro* reaction between DAO and MeS-D-KYN (*5*). In addition, DAO activity was fluorometrically detected by fluorescence microscopy in cultured LLC-PK1 cells incubated with MeS-D-KYN (*5*). However, there are few reports on the occurrence of DAO activity in mammalian tissues *in vivo* under alive condition. Therefore, we investigated DAO activity *in vivo* using the microdialysis (MD) technique (*6–8*), which has been widely used in research on metabolic enzymes (*7*). This technique involves a dialysis membrane that is embedded in the tissue of an experimental animal.

The embedded membrane is permeable to small molecules, such as endogenous compounds or drugs, enabling the monitoring of alterations in the levels of drugs or endogenous compounds in the tissues of live experimental animals, by analyzing the molecules that pass through the dialysis membrane.

Thus, the present study aimed to determine the potential of MeS-D-KYN in evaluating DAO activity *in vivo* using the MD technique in rats (Scheme 2). As relatively high DAO activity has been reported in the rat kidney (*9*), it was selected as the target organ in the present study.

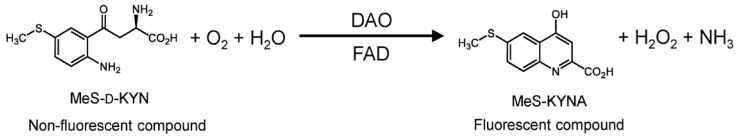
2. Material and Methods

2.1. Chemicals

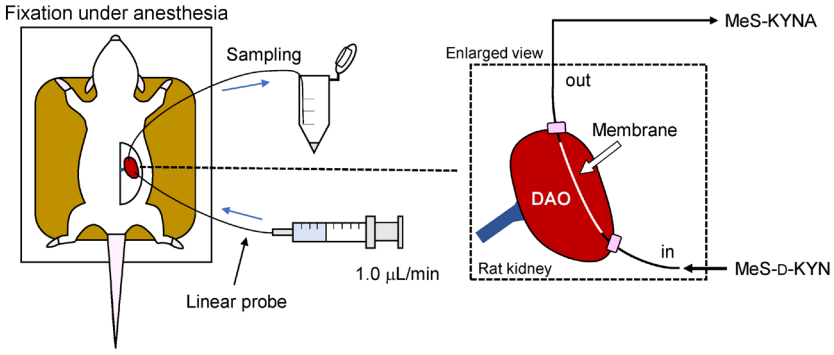
HPLC-grade methanol (MeOH), acetonitrile (CH_3CN), and formic acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 5-Chlorobenzo[*d*]isoxazol-3-ol (CBIO) was purchased from AstaTech, Inc. (Bristol, PA, USA). Sterilized Ringer's solution was purchased from Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Ketamine hydrochloride was purchased from FUJIFILM Wako Pure Chemical Corporation. Reduced L-glutathione and xylazine hydrochloride were obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). MeS-D-KYN and MeS-KYNA were synthesized in our laboratory according to a previous report (*5*).

2.2. Animal experiments

Animal experiments were approved by the Animal Care Committee of the Toho University (No. 21-55-369). Male Sprague-Dawley rats (7-8 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan) and housed in a temperature- and humidity-controlled room for at least one week before commencing the experiment. Renal MD operation was performed as previously described (*10*). Briefly, under anesthesia with an intra-muscular injection of ketamine/xylazine (90/10 mg/kg/mL in saline), the rat was laid supine on a heating pad (Bio Research Center Co., Ltd., Tokyo, Japan). The left kidney was exposed and a linear MD probe (10 mm membrane; BAS*i,* West Lafayette, IN, USA) was implanted inside it, with the dialysis membrane of the probe completely embedded in the kidney. The probe position was fixed using a hemostatic matrix, Integran® (Nippon Zoki Pharmaceutical Co., Ltd., Osaka, Japan) and surgical glue, aronarufa® (Daiichi-Sankyo Co., Ltd., Tokyo, Japan). One end of the linear probe was connected to a 1 mL gastight syringe, and Ringer's solution with 2.0 mM sodium phosphate at pH 7.0 and 5.0 mM glutathione was perfused *via* the syringe. The perfusion was performed at a rate of 1.0 µL/min using a syringe pump, 11 plus (Harvard apparatus, Holliston, MA, USA), 0-1.5 h and 3.5-6.5 h after initiating the renal MD experiment. Ringer's solution with 5.0 µM MeS-D-KYN was infused 1.5-3.5 h after starting the experiment (n = 4). For CBIO-infusion experiment, Ringer's solution with 10, 100, 500, and 2,500 µM CBIO was used as the perfusate 0-6.5 h after starting the experiment (n = 4 per concentration). For the oral CBIO administration experiment, 5.0 and 25 mg/kg CBIO was orally administered 1.5 h after starting the experiment (n = 4 per concentration). MD samples or



Scheme 1. The fluorescent compound MeS-KYNA is enzymatically produced from the non-fluorescent compound MeS-D-KYN by the action of DAO in the presence of FAD.



Scheme 2. Illustration of rat renal microdialysis experiment in the present study.

dialysates (approximately 30 µL) were collected every 0.5 h under ice-cold conditions. The first three samples, collected over a period of 1.5 h, were used as baseline samples.

2.3. HPLC

HPLC was conducted using an AS-4050*i* autosampler, PU-4180 intelligent pump, CO-2065 *plus* column oven (Jasco Corporation, Tokyo, Japan), and L-2485 fluorescence detector (Hitachi High-technologies, Tokyo, Japan). Inert Sustain®C18 (4.6 × 250 mm, 5.0 µm; GL Sciences Inc., Tokyo, Japan) separation column set at 40°C in the column oven was used. Mobile phases A and B were 0.05% HCO_2H in H_2O and 0.05% HCO_2H in CH_3OH, respectively, and were eluted following a gradient time-program: 0-5 min, B% = 3; 5-30 min, B% = 3-100; 30-45 min, B% = 100; and 45-60 min for initializing for next analysis. The fluorescence detection wavelength was set at 450 nm with an excitation wavelength of 364 nm. The obtained chromatograms were analyzed using ChromNavi® software (Jasco).

2.4. Statistical analysis

Data are expressed as mean ± standard error (S.E.). For comparison between the two groups, the Mann-Whitney *U*-test was used. For comparison among the three groups, a one-way analysis of variance (ANOVA) followed by the Bonferroni test was used. A p-value < 0.05 was considered statistically significant.

3. Results and Discussion

The present study evaluated the *in vivo* DAO activity through a renal MD experiment in rats. The dialysates were evaluated before (0-1.5 h), during (1.5-3.5 h), and without (3.5-6.5 h) MeS-D-KYN. The samples collected every 30 min were immediately analyzed using a reversed-phase HPLC with fluorescence detection.

A peak of standard MeS-KYNA was fluorometrically detected at approximately 22 min (Figure 1a). MeS-KYNA was barely observed in the samples before infusion of MeS-D-KYN (Figure 1b), indicating that MeS-KYNA is not an endogenous compound in rat kidneys. After infusion of 5.0 µM MeS-D-KYN, the MeS-KYNA peak was gradually observed (Figure 1c). Upon stopping the infusion of MeS-D-KYN, the MeS-KYNA peak disappeared (Figure 1d).

In addition, we previously performed MD with D-KYN infusion at 1.0 µL/min in the rat prefrontal cortex for the evaluation of DAO activity *in vivo* (*11*). In this case, the metabolite KYNA, an endogenous compound, was produced by the action of DAO. Therefore, to detect endogenous KYNA, a column-switching HPLC-fluorescence detection system (*12*), which employed a post-column reaction device with flowing 200 mM zinc ion (Zn^{2+}), was used; owing to the weak fluorescence of KYNA, Zn^{2+} addition is necessary for the formation of KYNA-Zn^{2+} fluorescence complex (*13*). This complex is usually detected by HPLC, where Zn^{2+} is added to the mobile phase, to determine the KYNA content (*14,15*). In contrast to these previous studies, in the present study, the conventional HPLC-fluorescence detection method could detect MeS-KYNA owing to the intense MeS-KYNA fluorescence that was observed without Zn^{2+} addition. The fluorescence of MeS-KYNA was approximately 200 times more intense than that of KYNA (*5*). As shown in Figure 2a, the fluorescent peak area of MeS-KYNA in the MD samples was observed at 1.0-1.5 h after starting the infusion of 2.5 or 5.0 µM MeS-D-KYN. Based on these data, the area under the curve (AUC) values for 2.5 and 5.0 µM MeS-D-KYN infusion were calculated and compared. The AUC for the 2.5 µM infusion was approximately half of that for the 5.0 µM infusion (Figure 2b), suggesting a concentration-dependent production of MeS-KYNA from MeS-D-KYN. These results clearly indicate that MeS-KYNA was produced from MeS-D-KYN in the rat kidneys. Briefly, MeS-D-KYN dissolved in Ringer's solution penetrated the rat renal cells through the MD membrane where it was oxidatively deaminated to MeS-KYNA by DAO (Scheme 2).

In addition, to clarify the involvement of renal DAO in the production of MeS-KYNA from MeS-D-KYN, a commercial inhibitor of DAO, CBIO, was continually co-infused with MeS-D-KYN 0-6.5 h after starting the MD experiment. As shown in Figure 3a,

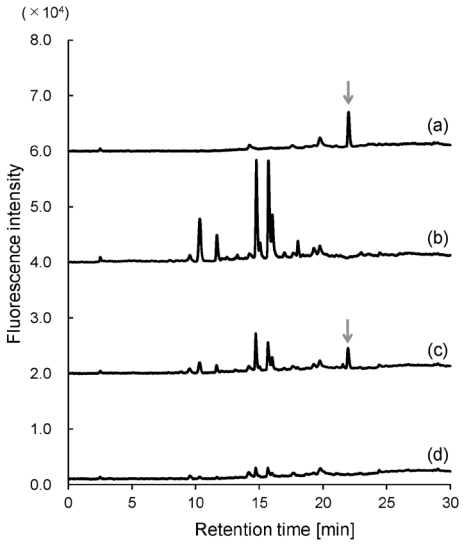


Figure 1. Chromatograms of standard MeS-KYNA and renal MD samples. (**a**) Standard MeS-KYNA and (b-d) renal MD samples on ODS column; (**b**) Before infusion of MeS-D-KYN (0-0.5 h); (**c**) During infusion of MeS-D-KYN (2.0-2.5 h), and (**d**) During infusion without MeS-D-KYN (4.0-4.5 h). The arrow indicates the peak of MeS-KYNA.

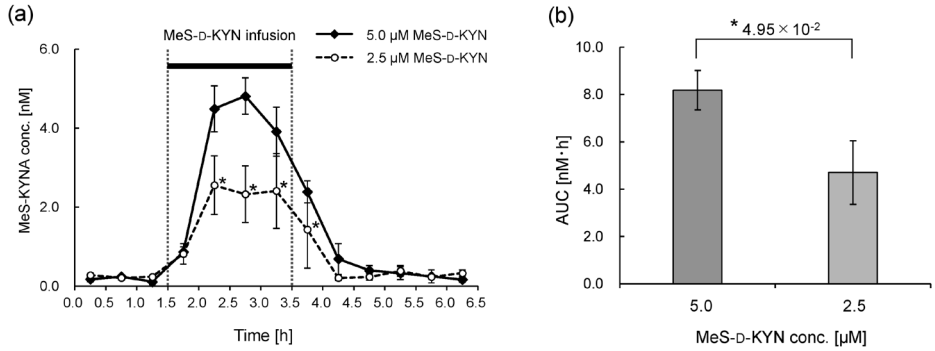


Figure 2. Changes in MeS-KYNA concentration in rat renal MD samples during infusion of 2.5 and 5.0 μM MeS-D-KYN (*n* = 4). (**a**) Time-course changes in MeS-KYNA concentration in rat renal MD samples. Open and closed circles with dotted and solid lines indicate the profiles during the infusion of 2.5 and 5.0 μM MeS-D-KYN, respectively (*n* = 4). (**b**) Area under the curves (AUCs) for MeS-KYNA during infusion of 2.5 and 5.0 μM MeS-D-KYN (*n* = 4). *$p < 0.05$.

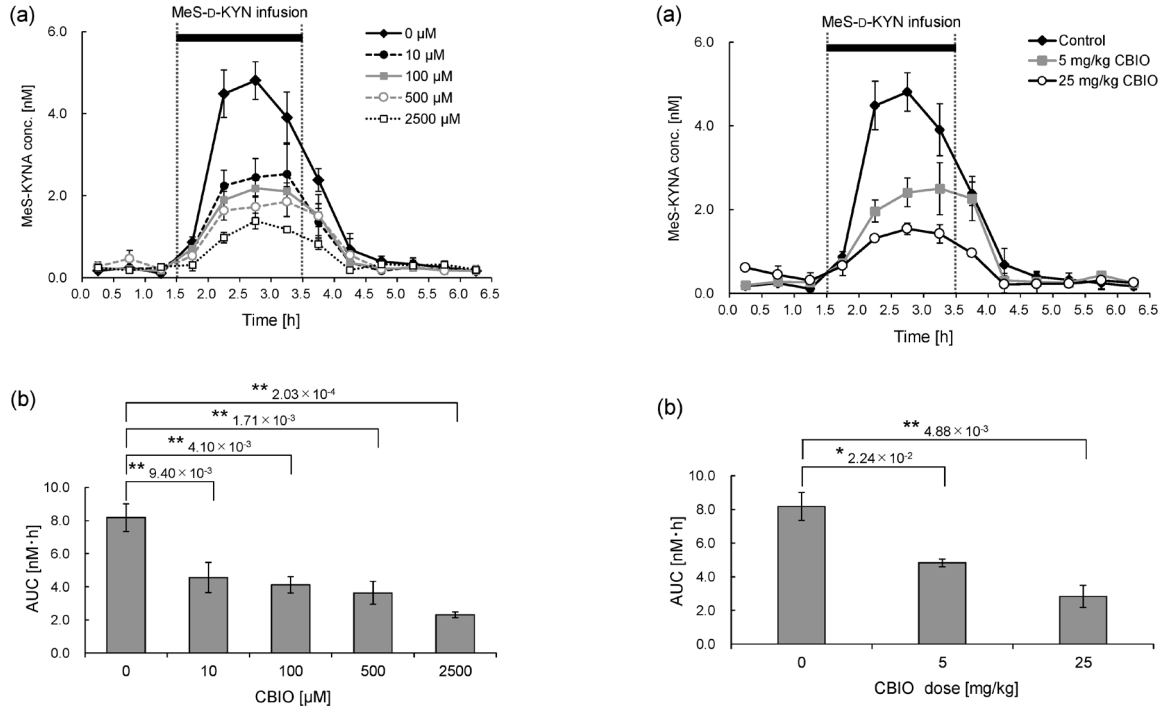


Figure 3. Changes in MeS-KYNA concentration in rat renal MD samples during infusion of 5.0 μM MeS-D-KYN in the presence of 0, 10, 100, 500, and 2,500 μM CBIO in Ringer's solution (*n* = 4). (**a**) Time-course changes in MeS-KYNA concentration in rat renal MD samples during infusion of 5.0 μM MeS-D-KYN and co-infusion of 0, 10, 100, 500, and 2,500 μM DAO inhibitor, CBIO (*n* = 4). (**b**) Area under the curves (AUCs) for MeS-KYNA during infusion of 5.0 μM MeS-D-KYN in the presence of 0, 10, 100, 500, and 2,500 μM CBIO in Ringer's solution (*n* = 4). **$p < 0.01$.

Figure 4. Changes in MeS-KYNA concentrations in rat renal MD samples during the infusion of 5.0 μM MeS-D-KYN after oral administration of CBIO at a dose of 0, 5, and 25 mg/kg (*n* = 4). (**a**) Time-course changes in MeS-KYNA concentrations in rat renal MD samples during the infusion of 5.0 μM MeS-D-KYN. Closed circles, closed squares, and open circles indicate the profiles after oral administration of DAO inhibitor CBIO at a dose of 0, 5, and 25 mg/kg (*n* = 4). (b) Area under the curves (AUCs) for MeS-KYNA during infusion of 5.0 μM MeS-D-KYN after oral administration of CBIO at a dose of 0, 5, and 25 mg/kg (*n* = 4). *$p < 0.05$, **$p < 0.01$.

CBIO remarkably suppressed the production of MeS-KYNA from MeS-D-KYN in a concentration-dependent manner. CBIO, at a concentration > 10 μM, suppressed the production of MeS-KYNA by approximately 50%. As CBIO is a small molecule, it may have penetrated the MD membrane to reach rat renal cells to directly inhibit renal DAO activity. After 2,500 μM CBIO co-infusion, MeS-KYNA levels decreased by approximately 20% compared to those in the non-infused group (Figure 3b). Taken together, these results indicate that MeS-D-KYN was oxidatively metabolized mainly by DAO, but enzymes other than DAO may also be involved in the oxidation of MeS-D-KYN.

We also investigated the influence of CBIO on renal

DAO activity by orally administering the compound to rats. At oral doses of 5.0 and 25 mg/kg, the AUCs for MeS-KYNA production were significantly decreased to approximately 60% and 35% compared to those of control (0 mg/kg), respectively. Thus, oral CBIO administration reduced the production of MeS-KYNA in a dose-dependent manner (Figures 4a and 4b). In this case, although CBIO reached the rat kidneys *via* the circulatory system, passing through the intestine and liver, it clearly inhibited DAO. Oral CBIO administration at 30 mg/kg was reported to be pharmacologically active in *in vivo* mouse behavior experiments, as the co-administration of D-serine (30 mg/kg) increases extracellular D-serine levels to attenuate pre-pulse inhibition deficits induced by dizocilpine (*16*). Although a dose of CBIO, 30 mg/kg, may be required for *in vivo* behavior experiments, such pre-pulse inhibition, the present study demonstrated that 5.0 mg/kg CBIO could suppress DAO activity in rat kidneys.

Currently, DAO is one of the target enzymes for developing novel drugs to overcome schizophrenia (*17,18*). The MD technique described in the present study using MeS-D-KYN can be used for evaluating drug candidates to inhibit DAO activity *in vivo* following oral administration.

4. Conclusion

The present study demonstrated the DAO-mediated production of MeS-KYNA from MeS-D-KYN *in vivo* using renal MD experiments in rats. This method, wherein MD is coupled with continuous MeS-D-KYN infusion, will be useful for evaluating *in vivo* DAO activity in tissues of live experimental animals.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

Ethical approval: Animal experiments were approved by the Animal Care Committee of the Toho University (No. 21-55-369).

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