Volume 331, number 1,2, 4–8 FEBS 12997 September 1993

Widespread distribution of free D-aspartate in rat periphery

Atsushi Hashimoto[a,b,*], Toru Nishikawa[b], Takae Oka[b], Tokishi Hayashi[d], Kiyohisa Takahashi[b,c]

[a]*Group of Cell and Information, Precursory Research for Embryonic Science and Technology (PRESTO), Research Development Corporation of Japan, 2-5-2, Nagata-cho, Chiyoda-ku, Tokyo 100, Japan*
[b]*Department of Mental Disorder Research, National Institute of Neuroscience and*
[c]*National Center Hospital for Mental, Nervous and Muscular Disorder, NCNP, 4-1-1, Ogawa-Higashi, Kodaira-shi, Tokyo 187, Japan*
[d]*Grelan Pharmaceutical Co Ltd., 2-12-3, Sakurashinmachi, Setagaya-ku, Tokyo 154, Japan*

Received 21 July 1993; revised version received 2 August 1993

We have identified and quantified free D-aspartate in adult rat peripheral organs using gas chromatographic-mass spectrometric and high-performance liquid chromatographic techniques. The level of free D-aspartate was highest in the adrenal, testis, spleen and pituitary, followed by the thymus, lung, ovary, placenta, pancreas and heart, and below the detection limit in the kidney, liver, brain, muscle and serum. These data provide the first evidence that a high level of free D-aspartate widely occurs in the adult rat periphery and suggest that the D-amino acid may be an endogenous substrate for D-aspartate oxidase.

D-Aspartate; D-Aspartate oxidase; D-Serine; D-Enantiomer; Rat periphery

1. INTRODUCTION

It has long been assumed that L-forms of amino acids exclusively constitute free amino acid pools in mammalian tissues [1]. However, recent evidence has demonstrated that free D-aspartate occurs in the developing central nervous system (CNS) and adult pituitary of chickens, rodents and humans at markedly high concentrations [2–4], and a persistently high level of free D-serine exists in rodents and human CNS tissues throughout life [4–8]. Furthermore, the anatomical distribution of CNS D-serine is closely correlated with that of the *N*-methyl-D-aspartate (NMDA)-type excitatory amino acid receptor-associated recognition sites [7]. Because D-aspartate and D-serine are well characterized to potentiate the NMDA receptor-mediated responses by selective stimulation of the excitatory amino acid recognition site and the strychnine-insensitive glycine recognition site, respectively [9–11], it is proposed that these D-forms of amino acids are novel candidates as intrinsic ligands for the NMDA receptor-related recognition sites in the brain [4–8].

Recently, a high-affinity L-glutamate/L-aspartate transport system has been cloned and characterized in mammalian CNS and periphery [12,13]. Like L-glutamate and L-aspartate, D-aspartate is also shown to be a selective substrate for this system [12,13]. Furthermore, D-aspartate oxidase, which selectively catalyzes the oxidative deamination of D-aspartate, is phylogenetically conserved in mammalian CNS and periphery [14–18], although the endogenous substrate for this enzyme so far has not been established [15]. Thus, these facts suggest that not only the pituitary but also other peripheral tissues may contain free D-aspartate. In order to explore this possibility, we identified and quantified free D-aspartate in adult rat peripheral organs using gas chromatographic (GC)-mass spectrometric (MS) and high-performance liquid chromatographic (HPLC) techniques.

*Corresponding author. Address: Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawa-Higashi, Kodaira, Tokyo 187, Japan. Fax: (81) (423) 46 1744

A. Hashimoto is a Visiting Researcher at the National Institute of Neuroscience from Group of Cell and Information, Precursory Research for Embryonic Science and Technology (PRESTO), Research Development Corporation of Japan, 2-5-2, Nagata-cho, Chiyoda-ku, Tokyo 100, Japan.

Abbreviations Boc-L-Cys, *N*-tert-butyloxycarbonyl-L-cysteine; CNS, central nervous system; D-HCA, D-homocysteic acid; EI, electron-impact ionization; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry, NMDA, *N*-methyl-D-aspartate; OPA, *o*-phthaldialdehyde; PFPA, pentafluoropropionic anhydride; TCA, trichloroacetic acid.

2. MATERIALS AND METHODS

2.1. *Reagents*

All amino acids except D-homocysteic acid (D-HCA), trichloroacetic acid (TCA), glacial acetic acid and *o*-phthaldialdehyde (OPA) were obtained from Nacarai Tesque (Japan). D-HCA, *N*-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys), pentafluoropropionic anhydride (PFPA) and all solvents of HPLC grade were purchased from Sigma (USA), Novabiochem (Switzerland), G.L.C Science (Japan) and Kanto Chemicals (Japan), respectively

 Published by Elsevier Science Publishers B V

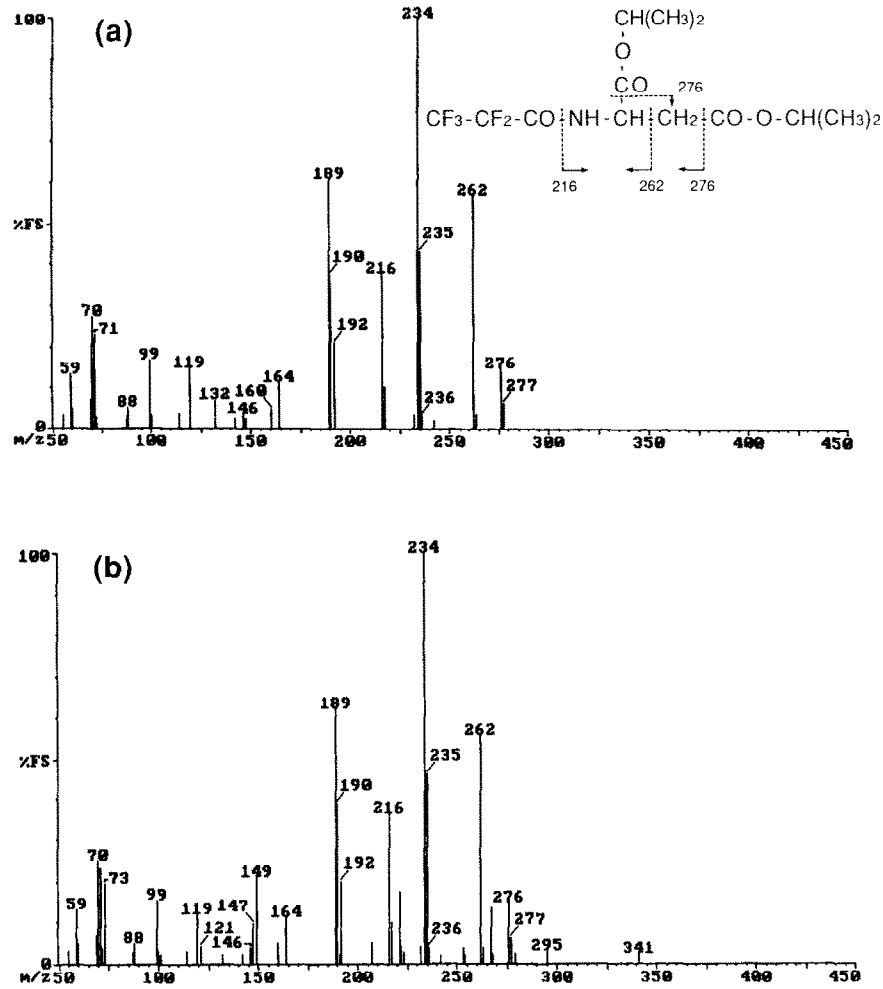

Fig. 1. Electron-impact ionization mass spectra of N-PFP-isopropyl derivative of authentic D-aspartate (a) and the compound, which co-eluted with the authentic D-aspartate derivative, in the testis extract (b).

2.2. *Animals and tissue preparation*

Male and female rats (Clea Japan, Japan) weighing 160–170 g at the time of the experiments were used. The animals were housed at 22.0 ± 0.5°C in a humidity-controlled room under a 12-h light–dark cycle (light on at 8.00 h) and were allowed food and water ad libitum. The animal was perfused with physiological saline under pentobarbital anesthesia (40 mg/kg, intraperitoneally) to eliminate contamination by amino acids in the blood. The perfused tissue sample was homogenized in 10 vols. of 5% TCA after the addition of D-HCA, and the homogenate was centrifuged at 18,000 × *g* for 30 min at 4°C. To remove TCA, the supernatant was extracted three times with water-saturated diethyl ether. The aqueous layer was then passed through a Millipore filter, HV (0.45 μm) and stored at −80°C until derivatization.

2.3. *GC-MS analysis*

The electron-impact ionization (EI)-MS analysis was performed on a Fisons VG MD-800 mass spectrometer (Jasco, International Co., Japan), equipped with a Hewlett-Packard 5890A gas chromatograph (Hewlett-Packard, Japan) and a Chirasil-L-Val capillary column (25 m × 0.25 mm, Gasukuro Kogyo, Japan) as previously described [5]. After the TCA extract was derivatized with isopropanol/2 N HCl and PFPA, the N-PFP-isopropyl amino acid derivative was injected into the gas chromatograph.

2.4. *HPLC analysis*

The simultaneous determination of free amino acid enantiomers and non-chiral amino acids in the rat tissues was accomplished by HPLC equipped with a fluorometric detector and a 4-μm Nova-Pak C18 column (Waters, Japan) as previously described [6]. Briefly, the TCA extract was derivatized with Boc-L-Cys and OPA for 2 min at room temperature, and the derivatized sample was immediately applied to the HPLC system.

3. RESULTS

GC-MS analysis was performed in order to demonstrate the presence of free D-aspartate in rat periphery. Two distinct peaks, which showed the same retention times as the N-PFP-isopropyl derivatives of authentic D- (14.9 min) and L-aspartate (15.2 min), respectively, were observed on a gas chromatogram of a rat testis sample (data not shown). Fig. 1a and b shows the EI mass spectra of the authentic D-aspartate (a) and the compound, which co-eluted with the authentic D-aspartate, in the testis extract (b). The EI mass spectrum of

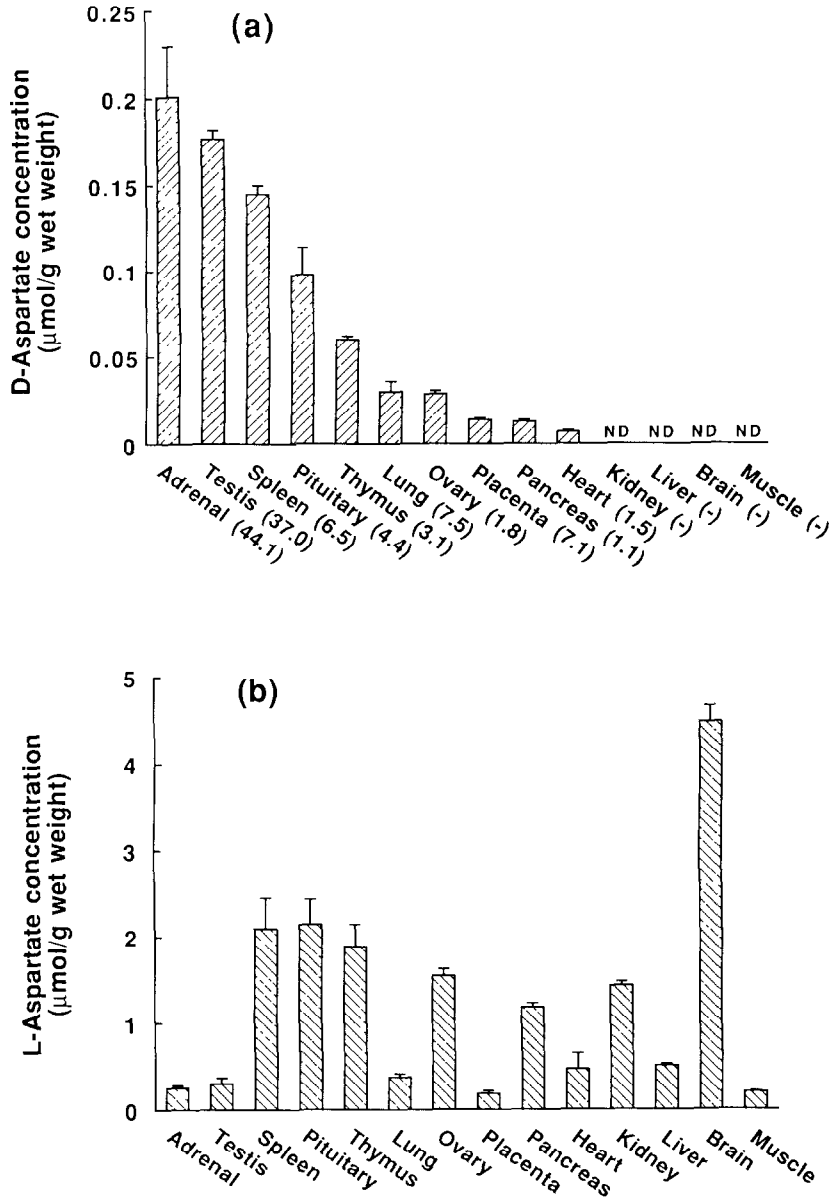

Fig. 2. Tissue distribution of free D-aspartate (a) and L-aspartate (b) in 7-week-old rats. The ratios of D- to total (D + L) aspartate are expressed as percentages in parentheses. Values represent means with S.E.M of three to six samples. ND, not detectable.

the authentic D-aspartate displayed a base peak at *m/z* 234 $[CF_3CF_2CONHCHCH_2COOH]^+$ and/or $[CF_3CF_2CONHCH(CH_2)COOH]^+$ and four prominent fragment peaks at *m/z* 189 $[CF_3CF_2CONHCHCH_2]^+$, at *m/z* 216 $[(CH_3)_2CHOCOCH(NH)CH_2COOCH(CH_3)_2]^+$, at *m/z* 262 $[CF_3CF_2CONHCHCOOCH(CH_3)_2]^+$ and at *m/z* 276 $[CF_3CF_2CONHCH(CH_2)COOCH(CH_3)_2]^+$ and/or $[CF_3CF_2CONHCHCH_2COOCH(CH_3)_2]^+$ (Fig. 1a). Among them, the peak at *m/z* 216, which is characteristic of the structure of the aspartate derivative, was also found in the spectrum of the compound in the testis extract (Fig. 1b). Although some weak fragment peaks, which are considered to be derived from impurities in the sample, were present in the EI-mass spectrum of the compound, the spectrum was almost identical to that of the authentic D-aspartate (Fig. 1b). The spectrum of the compound, which co-eluted with the authentic L-aspartate, also contained these characteristic fragment peaks (data not shown). Consequently, these data prove that these two compounds of the testis extract are D- and L-aspartate, respectively.

The concentrations of free D- and L-aspartate in rat peripheral tissues were estimated using the HPLC technique as previously reported [6]. As shown in Fig. 2a, D-aspartate was unevenly distributed in the peripheries of a 7-week-old rat. The content of free D-aspartate and the ratio of D- to total aspartate were highest in the adrenal (0.20 ± 0.03 μmol/g wet weight, 44.1%, $n = 3$).

Higher contents were seen in the adrenal, testis, spleen and pituitary, followed by the thymus, lung, ovary, placenta, pancreas and heart, and under the detection limit in the kidney, liver, brain and femoral muscle (<0.01 μmol/g wet weight). In contrast, L- aspartate ubiquitously occurred in these peripheral tissues and the regional variation of L-aspartate was entirely different from that of D-aspartate (Fig. 2b). The level of L-aspartate was higher in the brain, pituitary, spleen, thymus, ovary, kidney and pancreas, with decreasing levels in the liver, heart, lung, testis, adrenal, placenta and femoral muscle. The serum contents of D- and L-aspartate were below the detection limit and 45.0 ± 3.7 (nmol/ml, $n = 6$), respectively. There was no significant correlation between the D-aspartate content and the L-aspartate content in these organs.

The trace or low levels of D-serine were also demonstrated in these tissues except the brain tissue (unpublished data). Furthermore, D-glutamate and the other D-amino acids were below the detection limit (<0.01 μmol/g wet weight).

4. DISCUSSION

The present results demonstrate that a peak, which exhibited the identical retention time as the N-PFP-isopropyl authentic D-aspartate derivative, is detected in the rat testis and the EI mass spectrum of the peak of the testis extract is almost identical to that of the authentic D-aspartate. We further demonstrate that a high content of D-aspartate is widely present in the adult rat periphery. A markedly high level of D-aspartate in the pituitary and non-detectable levels of the D-form in the kidney, liver, brain and serum correspond well with previous reports [2,3,7,8]. The accuracy of quantification of D-aspartate was further confirmed by the observation that a similar distributional profile was demonstrated in rat tissues by refined HPLC determination after derivatization with *N*-acetyl-L-cysteine and OPA [19] (data not shown).

The characteristic distributional profile of D-aspartate might solely reflect regional activity of D-aspartate oxidase or regional variation of the L-glutamate/L-aspartate transport system. However, this is unlikely because, (a) although the activity of D-aspartate oxidase is known to be high in the kidney, liver and brain and below the detection limit in the heart and serum [16], the level of D-aspartate is at a trace or a non-detectable level in these organs (the present study) and (b) despite the predominant expression of this transporter mRNA in kidney and brain [13], the levels of the D-amino acid are below the detection limit in these organs. In contrast, it seems likely that D-aspartate oxidase may participate in the catabolism of the D-amino acid in at least the CNS, because the D-aspartate content dramatically declines to a trace level by postnatal day 10 in rat brain [2], while CNS D-aspartate oxidase activity increases rapidly from birth to postnatal day 10 or week 4 [16,17].

The source and metabolic system of peripheral D-aspartate remain to be resolved. Free D-aspartate might originate from the diet or from intestinal bacteria. However, this possibility appears to be ruled out by the facts that the serum and total blood contents of free D-aspartate are only at trace levels [2] or below the detection limit in the present study and the absorption of amino acids from the small intestine prefers L- to D-amino acids [20,21]. Because the D-aspartyl residue is present in long-living proteins, free D-aspartate might be derived from the degradation of such molecules [22,23]. However, the fact that fetal blood and CNS in rats and humans contain a much higher content of D-aspartate than the adult blood and CNS conflicts with the above possibility [2–4], because the formation of D-amino acid in these proteins following post-translational racemization increases with advancing age or remains relatively constant during aging [22,23].

These observations, together with the fact that the total D-aspartate content of the chicken egg increases with the incubation period [3], provide further support for the possibility that de novo synthesis of D-aspartate occurs in mammalian CNS and peripheral tissues. Although aspartate racemase is proven to exist only in bacteria [24], it is plausible that this enzyme might be phylogenetically conserved in mammalian tissues and synthesize endogenous D-aspartate.

In conclusion, the present data demonstrate that a high level of free D-aspartate is extensively distributed in adult rat periphery and suggest that the D-amino acid may be an endogenous substrate for D-aspartate oxidase. Because the peaks of D-aspartate content in the heart, brain and retina of rats and chickens coincide well with the period of completion of organogenesis and/or functional maturation of each organ [3,25], free D-aspartate could play a pivotal role in the maturation of organs. Alternatively, because a lot of D-aspartate-rich organs such as adrenal, testis, pituitary, ovary and pancreas secrete a wide variety of hormones, the D-amino acid might be involved in the regulation of the endocrine system. Further study is required to elucidate the metabolic system and the physiological significance for free D-aspartate in mammals.

Acknowledgements: The authors wish to thank Mr. Y. Ohsawa and Mr. N. Harima for the measurements of mass spectra and for helpful comments (Jasco, International Co., Japan). This study was partly supported by research grants from the Research Development Corporation of Japan, the Ministry of Health and Wealth, and the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Corrigan, J.J. (1969) Science 164, 142–149.
[2] Dunlop, D.S., Neidle, A., MaHale, D., Dunlop, D.M. and Lajtha, A. (1986) Biochem. Biophys. Res. Commun. 141, 27–32.

[3] Neidle, A. and Dunlop, D.S. (1990) Life Sci. 46, 1517–1522.
[4] Hashimoto, A., Kumashiro, S., Nishikawa, T., Oka, T., Takahashi, K., Mito, T., Takashima, S., Doi, N., Mizutani, Y., Yamazaki, Y., Kaneko, T. and Ootomo, E (1993) J. Neurochem. 61, 348–351
[5] Hashimoto, A., Nishikawa, T., Hayashi, T., Fujii, N., Harada, K., Oka, T and Takahashi, K. (1992) FEBS Lett. 296, 33–36.
[6] Hashimoto, A., Nishikawa, T., Oka, T., Takahashi, K and Hayashi, T. (1992) J. Chromatogr. 582, 41–48.
[7] Hashimoto, A., Nishikawa, T., Oka, T. and Takahashi, K. (1993) J Neurochem. 60, 783–786.
[8] Hashimoto, A., Nishikawa, T., Konno, R., Niwa, A., Yasumura, Y., Oka, T. and Takahashi, K. (1993) Neurosci. Lett. 152, 33–36.
[9] Ransom, R.W. and Stec, N.L. (1988) J. Neurochem. 51, 830–836.
[10] Kleckner, N W. and Dingledine, R (1988) Science 241, 835–837.
[11] Danysz, W., Fadda, E., Wroblewski, J.T. and Costa, E (1990) Life Sci 46, 155–164.
[12] Pines, G., Danbolt, N C., Bjøra°s, M., Zhang, Y., Bendaham, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E. and Kanner, B.I. (1992) Nature 360, 464–467.
[13] Kanai, Y. and Hediger, M.A (1992) Nature 360, 467–471.
[14] Yusko, S.C. and Neims, A.H (1973) J. Neurochem. 21, 1037–1039
[15] Hamilton, G.A. (1985) Adv Enzymol. 57, 85–178.
[16] Yamada, R., Nagasaki, H., Wakabayashi, Y. and Iwashima, A. (1988) Biochim. Biophys. Acta 965, 202–205.
[17] Van Veldhoven, P.P., Brees, C. and Mannaerts, G.P. (1991) Biochim. Biophys Acta 1073, 203–208.
[18] Kera, Y., Nagasaki, H., Iwashima, A. and Yamada, R. (1993) Comp. Biochem. Physiol. 104B, 739–742.
[19] Aswad, D.W. (1984) Anal. Biochem. 137, 405–409
[20] Gibson, Q.H. and Wiseman, G. (1951) Biochem J 48, 426–429.
[21] Finch, L.R. and Hird, F.J.R. (1960) Biochim. Biophys. Acta 43, 278–287.
[22] Man, E.H., Sandhouse, M.E., Burg, J. and Fisher, G.H. (1983) Science 220, 1407–1408
[23] Man, E.H., Fisher, G.H., Payan, I.L., Cadilla-Perezrios, R., Garcia, N M., Chemburkar, R., Arends, G. and Frey, W.H. (1987) J Neurochem 48, 510–515.
[24] Yohda, N., Okada, H. and Kumagai, H. (1991) Biochim. Biophys. Acta 1089, 234–240.
[25] Bivin, W.S., Crawford, M.P. and Brewer, N.R. (1980) in: The Laboratory Rat (Baker, H.J., Lindsey, J R. and Weisbroth, S H., Eds.) Vol. 1, pp. 74–100, Academic Press.