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Altered free amino acid levels in brain cortex tissues of mice with Alzheimer's disease as their *N*(*O*,*S*)-ethoxycarbonyl/tert-butyldimethylsilyl derivatives

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The altered amino acid (AA) levels as neurotransmitter closely correlate to neurodegenerative conditions including Alzheimer's disease (AD). Target profiling analysis of nineteen AAs in brain cortex samples from three Tg2576 mice as AD model and three littermate mice as control model was achieved as their N(O,S)-ethoxycarbonyl/tert-butyldimethylsilyl derivatives by gas chromatography. Subsequently, star pattern recognition analysis was performed on the brain AA levels of AD mice after normalization to the corresponding control median values. As compared to control mice, γ-aminobutyric acid among ten AAs found in brain samples was significantly reduced ($P < 0.01$) while leucine was significantly elevated ($P < 0.02$) in AD mice. The normalized AA levels of the three AD mice were transformed into distorted star patterns which was different from the decagonal shape of control median. The present method allowed visual discrimination of the three AD mice from the controls based on the ten normalized AA levels. [BMB reports 2008; 41(1): 23-28]

INTRODUCTION

The most common neurodegenerative disease, Alzheimer's disease (AD), is characterized by abnormalities in multiple brain regions and by progressive cognitive deterioration, including cognition, behavioral changes, and function (1-3). The etiology of this neurological disease is still unknown, but the identification of loss of cholinergic neurons in AD patients has advanced understanding of the molecular mechanisms of AD. Since AD is frequently misdiagnosed, it is important to identify surrogate outcome measure of biological markers for the disease. However, a few studies have investigated biomarkers for the disease (4-6).

The amino acids (AAs) such as aspartic acid, glutamic acid, γ-aminobutyric acid (GABA) and 3,4-dihydroxyphenylalanine (DOPA) are known to act as neurotransmitters in the central nervous system (4, 6-9). They play an important role in response to neurodegenerative conditions including AD (4, 6, 8, 10). AD is an age-related prevalent neurodegenerative disorder. The progressive cognitive decline and behavioral disturbances characterized by AD patients have been explained as neurochemical alterations of multiple neurotransmitters systems in brain tissue (4, 6-8, 11). Their accurate detection and measurement has become an important task in the study of etiology or progression of AD and other human neurodegenerative diseases.

Metabolic profiling analysis of multiple AAs require the use of high-resolution chromatography, mainly by high-resolution capillary gas chromatography (GC) (12-15) and high-performance liquid chromatography with fluorescence (7, 10, 16) or electrochemical detections (4, 6, 9, 11, 16). GC combined with mass spectrometry (MS) has been more preferred because of its inherent high resolving power, high sensitivity and positive peak confirmation. The accurate GC and GC-MS analyses, however, require one or more appropriate derivatization procedures to block all active protons present in AAs (17). For this purpose, alkylsilylation (18-23) and alkoxycarbonyl (AOC) esterification (24-27) methods have been widely used. The AOC reaction was frequently conducted in aqueous solution with subsequent esterification (28, 29) or alkylsilylation (30-35). In our recent report (35), two-phase ethoxycarbonyl (EOC) reaction in alkaline solution with ethyl chloroformate (ECF) present in dichloromethane phase with subsequent tert-butyldimethylsilyl (TBDMS) reaction was useful for the assay of clinically important AAs and carboxylic acids in a single run (35). However, this high pH conditions was not suitable for labile AAs such as 3,4-dihydroxyphenylalanine with catecholic hydroxyl group. Moreover, phenolic hydroxyl groups of AAs

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were partially derivatized into O-EOC derivatives prior to TBDMS derivatization. This problem could be circumvented by conducting two-phase EOC reaction with a pH shit from 8 to 12 as reported elsewhere for the labile catecholamines (36).

In continuation of the target metabolic analysis for diagnostic markers of AD (37, 38), the present investigation was undertaken to measure the levels of free AAs as the neurochemicals in brain cortex specimens from three control mice and three diseased mice with AD as the animal models. In this study, the EOC reaction conducted in one-step in strong alkaline solution (35) was modified into two-step pH shift mode (initially at pH 8 then raised to pH 12) for the simultaneous analysis of 19 AAs including 3,4-dihydroxyphenylalanine as their N(O,S)-EOC/ TBDMS derivatives. Star symbol plotting as the visual pattern recognition method (38, 39) was employed to convert the quantitative differences in the AA levels between control group and AD mice into decagonal shapes.

RESULTS AND DISCUSSION

Levels of amino acids in brain cortex tissues from AD mouse models

Upon GC analysis of a control brain cortex spiked with the nineteen AAs, each AA including labile DOPA as their respective EOC/TBDMS derivatives was readily detected along with the biogenic AAs present in the tissue sample (Fig. 1-upper). Among the nineteen AAs searched, a total of ten neurochemical AAs were screened from the six mice examined as exemplified by a control mouse (Fig. 1-middle) and an AD mouse (Fig. 1-bottom). DOPA was not found from any of the brain samples. Each AA was positively identified by GC-MS analysis.

The AA levels measured in minimal brain cortex tissues (5 mg) of the six mice ranged from 0.020 to 1.155 μg/mg of brain cortex tissue with good repeatability (Table 1). The amounts of threonine (No. 6), serine (No. 7) and GABA (No. 8) were calculated on sum of mono-EOC/mono-TBDMS and mono-EOC/di-TBDMS derivatives. In the control mice, GABA was the most abundant, followed by glutamic acid, serine, threonine and aspartic acid. Leucine was the least. Relatively small variations in the AA levels were noticed among the three control mice. In contrast, large variations in the levels of aspartic acid and glutamic acid were noticed among the AD mice. In AD-1 case, glutamic acid was the most abundant, followed by GABA, aspartic acid, threonine and serine. For the AD-2 and AD-3 mice, GABA was the largest but other AAs showed different orders in their levels. The large variations observed among the three Tg2576 mice might be due to the individual differences in the progress of AD caused by the different genetic make-ups and metabolic rates. According to the Student's t-test on the control mean and AD mean values, the levels of GABA were significantly reduced ($P < 0.01$) while the levels

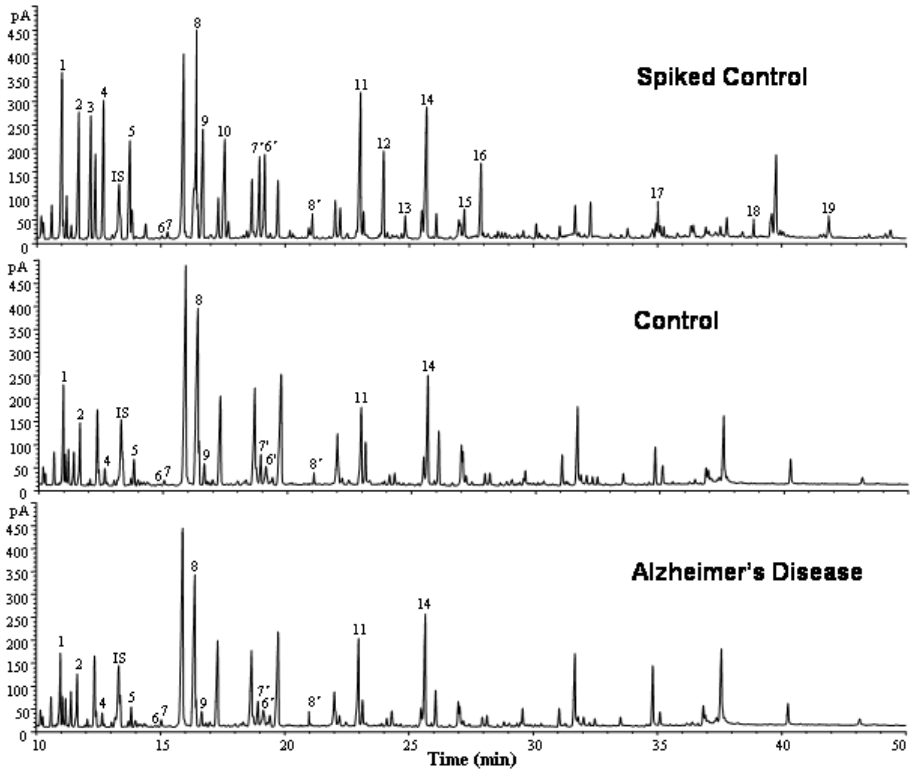

Fig. 1. GC profiles of amino acids as N(O)-ethoxycarbonyl/tert-butyldimethylsilyl derivatives in control brain cortex spiked with nineteen amino acids (Spiked Control), brain cortex tissues of a control mouse (Control) and a Tg2576 mouse (Alzheimer's Disease). Peaks: (1) alanine, (2) glycine, (3) α-aminobutyric acid, (4) valine, (5) leucine, (6) threonine as mono-EOC/mono-TBDMS derivatives, (7) serine mono-EOC/mono-TBDMS derivatives, (8) GABA as mono-EOC/mono-TBDMS derivatives, (9) proline, (10) pipecolic acid, (7') serine as mono-EOC-di-TBDMS derivatives, (6() threonine as mono-EOC-di-TBDMS derivatives, (8) GABA as mono-EOC/di-TBDMS derivatives, (11) aspartic acid, (12) phenylalanine, (13) cysteine, (14) glutamic acid, (15) homocysteine, (16) α-aminioadipic acid, (17) tyrosine, (18) tryptophan, (19) 3,4-dihydroxyphenylalanine. IS-norvaline.

of leucine were markedly elevated ($P < 0.02$) in all three AD mice as compared to the control mice. However, glutamic acid as precursor of GABA in the enzymatic pathway involving glutamic acid decarboxylase and aspartic acid as excitatory neuro-transmitters within the central nervous system, and other AAs were not significantly different as compare to those of control group (Table 1). The significantly elevated leucine and reduced GABA levels may show prominent pathological changes such as neurodegeneration, neuromodulators and neuronal loss including disturbance of AA metabolism pool in the brain from AD model.

Star pattern recognition of AD mouse model

Upon normalization of levels of ten AAs in AD mice to the corresponding control median values, they were more informative by expressing the elevation or depletion of the AA levels in multiples (ranging from 0.420 to 2.090) of the control median values. When these normalized values were used as the variables to draw star graphs composed of ten rays, the differences between AD mice and control median were expressed as the visual star patterns (Fig. 2). Each AD pattern was distorted from the decagonal shape of control average. The common features in the star patterns were depicted by the size increase in leucine (ray 5) and aspartic acid (ray 11), and the reduction in alanine (ray 1) and GABA (ray 8). Unlike the most distorted pattern of AD-1, AD-2 and AD-3 showed similar patterns except for proline (ray 9) and glutamic acid (ray 14).

Although the number of animals (three in each group) was small in this study, the results were well defined by statistics analysis and were reproduced previous report that the level of GABA was reduced significantly in the postmortem brains with AD (4). Further specific analysis to clarify the significance of a

Table 1. Levels of amino acids found in brain cortex tissues from control mice and Tg2576 mice with AD

No.	Compound	Amino acid levels (mean ± SD) in μg/mg of brain cortex, n = 3						P-values *t*-test
		Control mouse model[a]			AD mouse model[b]			
		C-1	C-2	C-3	AD-1	AD-2	AD-3	
1	Alanine	0.115 ± 0.002	0.211 ± 0.009	0.220 ± 0.016	0.089 ± 0.003	0.185 ± 0.004	0.180 ± 0.007	NS[c]
2	Glycine	0.124 ± 0.008	0.153 ± 0.010	0.157 ± 0.013	0.133 ± 0.005	0.157 ± 0.014	0.154 ± 0.014	NS
4	Valine	0.020 ± 0.001	0.040 ± 0.002	0.040 ± 0.001	0.020 ± 0.001	0.046 ± 0.003	0.045 ± 0.002	NS
5	Leucine	0.025 ± 0.002	0.022 ± 0.002	0.026 ± 0.002	0.027 ± 0.001	0.032 ± 0.003	0.031 ± 0.003	0.02[d]
6	Threonine	0.530 ± 0.030	0.569 ± 0.013	0.660 ± 0.058	0.587 ± 0.021	0.575 ± 0.023	0.647 ± 0.008	NS
7	Serine	0.553 ± 0.041	0.591 ± 0.044	0.614 ± 0.036	0.573 ± 0.016	0.495 ± 0.024	0.615 ± 0.057	NS
8	GABA	1.046 ± 0.021	1.155 ± 0.027	1.056 ± 0.091	0.952 ± 0.032	0.894 ± 0.070	0.976 ± 0.046	0.01[d]
9	Proline	0.030 ± 0.002	0.047 ± 0.001	0.050 ± 0.005	0.026 ± 0.001	0.034 ± 0.002	0.049 ± 0.002	NS
11	Aspartic acid	0.350 ± 0.022	0.357 ± 0.029	0.324 ± 0.018	0.732 ± 0.067	0.439 ± 0.037	0.389 ± 0.034	NS
14	Glutamic acid	0.588 ± 0.033	0.499 ± 0.040	0.540 ± 0.033	1.047 ± 0.079	0.634 ± 0.057	0.455 ± 0.040	NS

[a] Amino acid levels in brain cortex tissues of three control mice (18 months, 3 males).
[b] Amino acid levels in brain cortex tissues of three mice with AD (18 months, 3 males).
[c] NS, not significant at 95% confidence level.
[d] Significantly different at 95% confidence level.

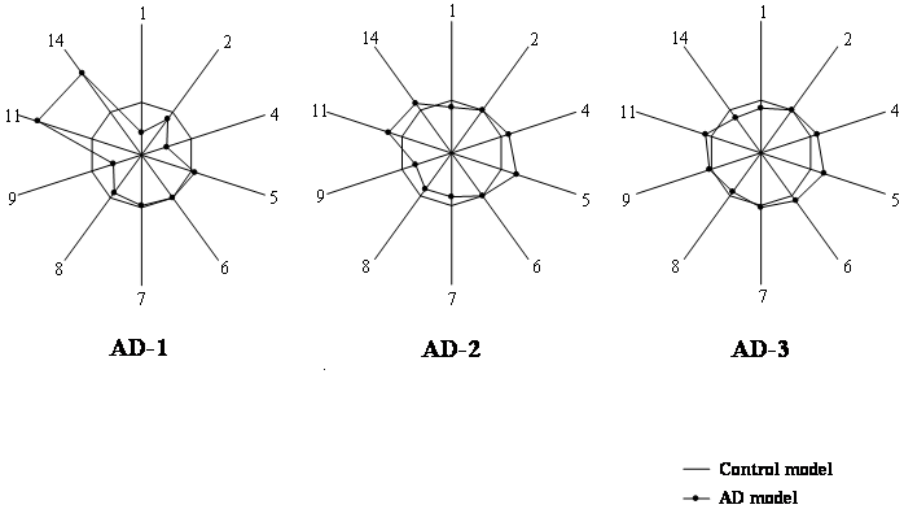

Fig. 2. Star symbol plots of three Tg2576 mice (AD-1, AD-2 and AD-3) with Alzheimer's disease based on the mean levels of the ten amino acids as the variables after normalization to the corresponding control median values. Rays: (1) alanine, (2) glycine, (4) valine, (5) leucine, (6) threonine, (7) serine, (8) GABA, (9) proline, (11) aspartic acid, (14) glutamic acid.

loss of neurons in AD will be needed in the other brain regions, such as hippocampus, thalamus, and basal ganglia.

In conclusions, the present AA profiling method provided simultaneous analysis of ten neurochemical AAs as N(O,S)-EOC/TBDMS derivatives in a minimal brain cortex tissue (5 mg) from each control and AD mouse model. When combined with star symbol plotting method, the ten normalized AA levels of AD mice were transformed into distorted star patterns, enabling their visual recognition from the decagonal shape of control mean. The present method might be useful for the visual discrimination of AD group from normal group.

MATERIALS AND METHODS

Chemicals and reagents

The twenty AAs including norvaline used as internal standard (IS) and ECF were purchased from various vendors such as Sigma-Aldrich. N-Methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). Toluene, diethyl ether, ethyl acetate and dichloromethane of pesticide grade were obtained from Kanto Chemical (Tokyo, Japan). Sulfuric acid and sodium hydroxide were from Duksan (Seoul, South Korea). All other chemicals were of analytical grade and used as received.

Preparation of standard solutions

Each stock solution of AAs was made up at 10 μg/μl in 0.1 M HCl. Their standard working solutions at 0.05 and 1.0 μg/μl were then prepared by diluting each stock solution with 0.1 M HCl. The IS stock solution prepared by dissolving norvaline at 10.0 μg/μl in 0.1 M HCl was used to make IS working solutions at 0.1 μg/μl in 0.1 M HCl. The mixed calibration samples were then prepared at five different concentrations ranged from 0.1 to 10.0 μg/μl by mixing appropriate aliquots of each working solution. All standard solutions prepared were stored at 4°C.

Instrumentation

The GC analyses were performed with an Agilent 6890 gas chromatograph, equipped with electronic pneumatic control system, a split/splitless inlet system, an automatic liquid sampler, two flame ionization detector system, Chemstation (Agilent Technologies, Atlanta, GA, USA). The injector was installed with an HP-50+ (OV-17 bonded) fused-silica capillary columns (30 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies, Atlanta, GA, USA). The injector and detector temperatures were 260 and 290°C, respectively. Helium was used as carrier gas at a flow rate of 1.0 ml/min with constant flow mode. Samples (ca. 1.0 μl) were injected in the splitless mode with purge delay time of 42 sec. The oven temperature was initially at 80°C for 1 min and programmed to 150°C at a rate of 20°C/min and finally to 290°C (10 min) at a rate of 4°C/min. All GC analyses were performed in triplicate.

GC-MS analyses were performed with an HP 5890 Aseries II gas chromatograph, interfaced to an HP 5970B mass-selective detector (70 eV, electron impact mode) and installed with an HP 50+ (OV-17 bonded) fused-silica capillary column (25 m × 0.20 mm I.D., 0.16 μm film thickness). The temperatures of injector and interface were 260 and 280°C, respectively. The inlet pressure of helium used as carrier gas was set to 85 kPa. Samples were introduced in the split-injection mode (10 : 1) and the oven temperature was initially at 100°C for 1 min and then programmed to 280°C at a rate of 4°C/min with holding time for 20 min. The mass range scanned was 50-650 u at a rate of 0.99 scans/s.

Sequential N(O,S)-ethoxycarbonyl/tert-butyldimethylsilyl derivatization

A mixed solution containing appropriate amounts (0.1-10.0 μg) of 19 AAs and fixed amount (1.0 μg) of norvaline as IS was taken into an aliquot (1 ml) of saturated sodium bicarbonate solution (pH 8.03). Two-phase EOC reaction was conducted by vortex-mixing (5 min) with ECF (5 μl) present in dichloromethane (1 ml) at room temperature. The aqueous phase was then adjusted to pH ≥ 12 (with 5.0 M NaOH) and immediately vortex-mixed (5 min) at room temperature after the second addition of ECF (10 μl). The aqueous solution was acidified (pH ≤ 2.0) (using concentrated sulfuric acid) with subsequent solvent extraction with diethyl ether (3 ml) and ethyl acetate (2 ml) in sequence. The combined extracts were evaporated to dryness under a gentle stream of nitrogen (40°C). Toluene (25 μl) and MTBSTFA (25 μl) were added to the residue containing EOC derivatives of AAs, and the mixture was heated at 60°C for 30 min to form TBDMS derivatives for GC and GC-MS analyses.

Sample preparation for assay of amino acids in brain cortex from AD mouse models

Three Tg2576 mice as AD model (AD-1, AD-2, AD-3, 18 months, male) and three littermate mice as control model (C-1, C-2, C-3, 18 months, male) (40) were used for this investigation. Each mouse was sacrificed by decapitation and the brain rapidly excised and the cortex was isolated and immediately snap-frozen in liquid nitrogen and stored at −80°C until being analyzed. A 20 mg of brain cortex tissue taken into 1 ml of distilled water containing norvaline (4 μg/ml) was homogenized (3 min, 30,000 rpm) in ice water bath with using a rotor/stator-type tissue homogenizer (Model Pro 200 Homogenizer, Wilenbrock Rd. Oxford, USA). An aliquot (equivalent to 5 mg brain tissue) of the homogenate was vortex-mixed with acetonitrile (0.25 ml) for 3 min. The mixture was diluted with 0.5 ml of sodium bicarbonate solution and centrifuged at 15,000 rpm (15 min) for protein precipitation. The supernatant layer was subjected to the aforementioned sequential EOC/TBDMS reactions for the GC and GC-MS analysis.

Star symbol plotting

The concentrations (μg/mg) of ten AAs positively found in

brain samples from control and AD mice were determined based on their calibration curves. Each AA level of the AD mice was normalized to the corresponding control median value. Each value was plotted as a line radiating from a common central point and the far ends of the lines were joined together to produce decagonal star patterns using MS Excel program as described elsewhere (38, 39).

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