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Analysis of trace amino acid neurotransmitters in hypothalamus of rats after exhausting exercise using microdialysis

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Abstract

A simple but effective coupling of microdialysis and capillary electrophoresis with laser induced fluorescence detection technique was applied to analysis of amino acid neurotransmitters in the hypothalamus of rats after acute exhausting exercise. The separation of amino acids was achieved using an uncoated fused-silica capillary (57 cm×75 μm I.D.) with a buffer of 10 m*M* disodium tetraborate at pH 10 and an applied voltage of 12.5 kV. The detection limit was 10^{-10} *M* for each amino acid. It is sufficiently sensitive and rapid for the determination of amino acids in a 5-μl Microdialysate. In comparison to pre-exercise, a significant increase in the levels of six hypothalamic amino acids (arginine, glycine, lysine, glutamic acid, alanine, γ-amino-*n*-butyric acid) was found after exercise. These results demonstrate that the increase of metabolic amino acids in the hypothalamus of rats can be induced by exhausting exercise and suggests that amino acid neurotransmitters may play functional roles in the central effects of exercise. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Microdialysis has gained wide recognition as a valuable tool for dynamic sampling of the extracellular space of living tissue [1,2]. The advantages and limitations of microdialysis have been discussed at length previously [3–6]. Briefly, a major advantage of the technique is that the microdialysate is completely free of proteins and other large biological debris, which eliminates the need for any additional sample clean-up procedures. This technique has been widely used for quantification of change in neurotransmitter concentrations in the extracellular space of the mammalian brain during behaviors, such as mating, sleeping and exercise [7–10].

E. Blomstrand et al. reported that exhausting exercise caused an increase of monoamine neurotransmitters (e.g. 5-Hydroxytryptamine, Dopamine and Noradrenaline) in hypothalamic area of rats and suggested that these changes may play an important role in the central effects of exercise [11]. The concentrations of amino acid neurotransmitters were a hundred times more than those of monoamine neurotransmitters in the brain [12]. It has not been established whether the increase of hypothalamic amine transmitters in response to exhaustive exercise is specific for this transmitter group or is also

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observed in the amino acid transmitters. In this work, microdialysis was applied to study the changes of the extracellular levels of hypothalamic amino acid neurotransmitters after acute exhausting exercise, in order to study the relationship between exercise fatigue and amino acid neurotransmitters.

Analysis of the neurochemical constituents of brain microdialysates has conventionally used high-performance liquid chromatography (HPLC) with electrochemical detection. However conventional HPLC analysis requires large injection volumes and hence lengthy dialysis sampling times. Capillary electrophoresis (CE) is a newly emerging technique for rapid and high-resolution separation of biological molecules [13]. Recently, it has been coupled with laser induced fluorescence detection for the separation and determination of amino acids [14–18]. Laser-induced fluorescence detection is one of the most sensitive detection techniques in CE because of the low background signal and the small sample volume required. Thus capillary electrophoresis combined with laser induced fluorescence detection is a technique well suited for the analysis of small-volume microdialysis samples.

In this paper, we report a rapid, sensitive method for analysis and detection of amino acid neurotransmitters in the hypothalamus of rats after exhausting exercise using microdialysis and capillary electrophoresis with laser-induced fluorescence detection.

2. Experimental

2.1. Chemicals

Unless stated otherwise, all chemicals were of analytical-reagent grade (Beijing Reagent Corp., Beijing). γ-Amino-*n*-butyric acid (GABA), alanine (ala), lysine (lys), argine (arg), glumate (gln), aspartate (asp), glysine (gly), glutamic acid (glu) and FITC were obtained from Sigma (St. Louis, MO, USA). Water was deionized and purified by a milli-Q millipore purification system (Millipore, Marlborough, MA, USA). The 5×10^{-4} *M* stock solutions of the amino acids were prepared in disodium tetraborate buffer and diluted to the desired concentration prior to use. The 5 m*M* stock solution of FITC was prepared in acetone.

2.2. Microdialysis

2.2.1. Animals

Adult male Sprague–Dawley rats with initial body weight of 250–300 g were used in this study and obtained from the laboratory animal center at the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). These animals were specific pathogen-free and were housed singly in cages with ad lib food and water. The rats anesthetized with ketamine hydrochloride (100 mg/kg, i.p.). A guide cannula was positioned above the left hypothalamus (AP: −0.8 mm, ML: 0.5 mm, DV: 6.7 mm).

2.2.2. Microdialysis procedure

Initially a microdialysis guide cannula was implanted above the left hypothalamus. Twenty four h after surgery a concentric design microdialysis probe, with a dialysis membrane 2 mm long and 0.2 mm O.D. with a molecular cut-off of 9000D (from Institute of chemistry, Chinese Academy of Sciences, Beijing), was implanted via the guide cannula into the hypothalamus area of the rat. The microdialysis probe was perfused with artificial cerebrospinal fluid (NaCl 120 m*M*, KCl 3.0 m*M*, $NaHCO_3$ 20 m*M*, $CaCl_2$ 1.2 m*M*, $MgCl_2$ 1.0 m*M*, Na_2HPO_4 0.25 m*M*, pH 7.4) via polyethylene tubing connected to a microinjection pump, at a flow rate of 1.5 μl/min. Following a 60 min stabilization period, perfusate fractions were collected every 15 min for 60 min. The probes remained in situ for 4 days. On the day of exercise, the rat was placed in a water tank and swam (loaded swimming of 6% body weight on tail of the rat) to exhaustion. The exhaustion index was that the rat could not float on water for 10 s. The rat was removed from the pool and the samples were collected. The same probe was used before and after exercise in the same rat. Recovery of microdialysis probes were tested in vitro, the results are shown in Fig. 1. The probe was repeatedly used nine times. The results showed that probe recovery from the second time of use to seventh time of use did not change appreciably. The microdialysis probes, which showed similar recoveries, were used in all experiments.

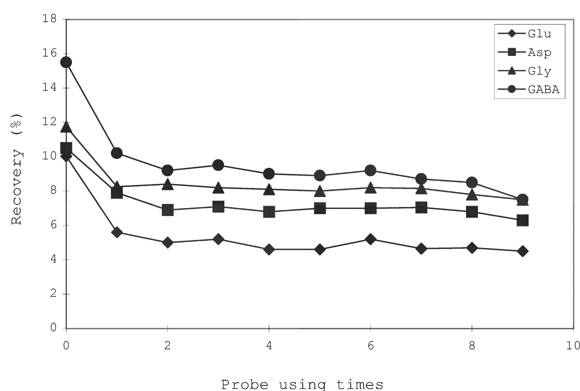

Fig. 1. Recovery of microdialysis probe in vitro (artificial cerebrospinal fiiud).

2.3. Capillary electrophoresis

2.3.1. Apparatus

All analysis were performed on a Beckman P/ACE 5500 system equipped with Beckman Laser Module 488 nm which consists of a 3 mW, 488 nm air-cooled Argon ion laser (Beckman-Fullerton CA, USA). Separations were performed in fused-silica capillary (57 cm×75 μm I.D.) (Beckman-Fullerton CA, USA) at 25°C, with a separation potential of 12.5 kV. The capillary was washed with 0.1 *M* NaOH, deionized water and separation buffer before use. All samples were injected pneumatically (0.5 p.s.i.) for 3 s.

2.3.2. Precolumn derivatization of standard amino acids

Precolumn derivatization of standard amino acid solutions were conducted in microvials. A final volume of 1.0 ml was used for each experiment. After appropriate amounts of the amino acid solution were diluted and mixed in 2 m*M* disodium tetraborate buffer at pH 10, FITC was added to give a final concentration thirty times higher than the total concentration of amino acid. Typically, the mixed solution was reacted at room temperature in the dark for 17 h. The derivatization solutions were then stored in the dark at 4°C before use.

2.3.3. Microdialysate derivatization

Five μl of the microdialysate was mixed with 10 μl 2 m*M* disodium tetraborate buffer and 5 μl 5×10^{-5} *M* FITC solution which contained 0.5% pyridine and left to stand for 17 h at room temperature in the dark. Before analysis, derivatization solutions were diluted with 80 μl 2 m*M* disodium tetraborate buffer.

2.4. Statistics

Conventional statistical methods have been applied to calculate mean values and standard errors of the mean. Differences have been tested for significance using student's *t*-test for paired observations.

3. Results and discussion

3.1. Separation condition

In our experiments, we investigated various separation buffers for the separation of amino acids. The result showed that disodium tetraborate buffer is a better resolution buffer to separate amino acid neurotransmitters. The pH values and concentration of borate buffer were optimized too. The optimum separation condition to separate amino acid was 10 m*M* disodium tetraborate buffer at pH 10 with a separation voltage of 12.5 kV. Figs. 2–4 show, respectively, typical electropherograms of the blank test, standard amino acid solutions and microdialysates. Peaks were identified by comparing migration times and spiking samples with known quantities of standard solution of amino acids. The

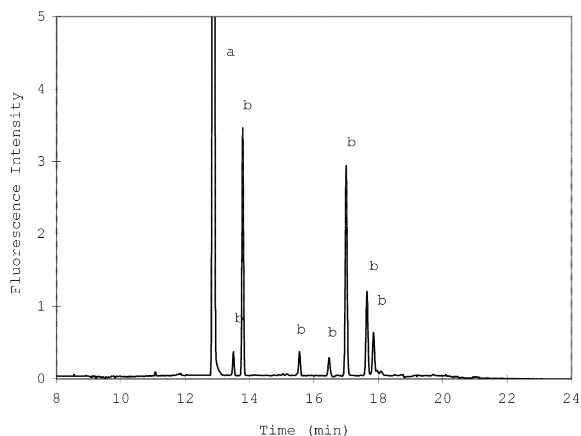

Fig. 2. Electropherogram of FITC in blank test. (a) FITC, (b) decomposition products of FITC.

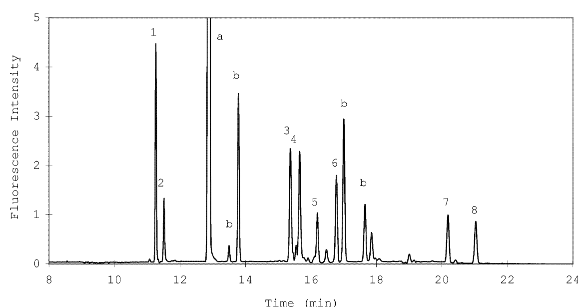

Fig. 3. Electropherogram of standard amino acids (each amino acids conc.: 2.5×10^{-8} *M*. (1) arg, (2) lys, (3) gln (4) GABA, (5) ala, (6) gly, (7) glu, (8) asp, (a) FITC, (b) decomposition products of FITC.

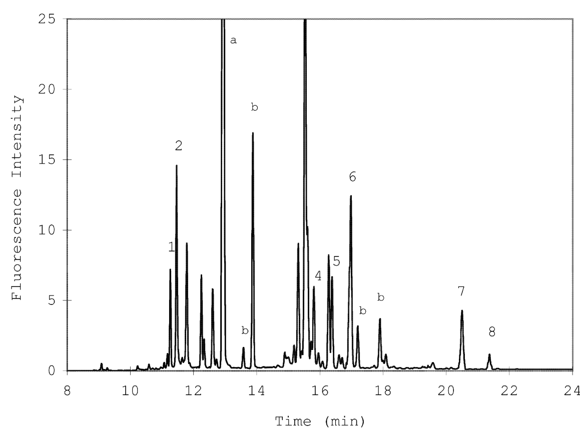

Fig. 4. Electropherogram of microdialysate, (1) arg, (2) lys, (3) gln, (4) GABA, (5) ala, (6) gly, (7) glu, (8) asp, (a) FITC, (b) decomposition products of FITC.

migration times of the analytes increase in the following order: (1) arg, (2) lys, (3) gln, (4) GABA, (5) ala, (6) gly, (7) glu and (8) asp. The migration time of the FITC-derivatized amino acid is strongly dependent on the electric charge that it carries and its mass.

3.2. Precision and limit of detection

The precision (expressed in term of relative standard deviation) of the present method for the various amino acids is summarized in Table 1. The relative standard deviations (RSD) of the migration times are typically less than 0.8%. The RSDs of the peak areas are typically in the range of 3.5–5.4%. The limits of detection for each amino acid (see Table 1) were estimated taking three times the standard deviation of the peak areas obtained from these solutions and calculating the corresponding concentration from seven repetitions. The detection limits were all $<7\times10^{-10}$ *M*, which was favorable for the analysis of microdialysates.

3.3. Linearity

The linearity of the present method was investigated by analyzing standard solutions containing a mixture of eight amino acids with known concentrations ranging from 5×10^{-9} to 2.5×10^{-7} *M*. From the electropherogram, the peak area is plotted against the concentration of the amino acid to obtain the calibration graph for each amino acid. The data points from the calibration graph were subjected to least-square regression analysis and the slope *a*, intercept *b* and correlation coefficients *r* for the various amino acids were calculated. The correlation coefficients of arg, lys, gln, GABA, ala, gly, glu, asp,

Table 1
Detection limits, elution times and RSD relation standard deviation of elution time and area for the various amino acid

Analyte	Detection limits (*M*)	Migration time (min)	RSD (%) (n=7)	
			Time	Area
Arg	9.4×10^{-11}	11.35	0.4	3.5
Lys	1.8×10^{-10}	11.55	0.4	4.5
Gln	2.2×10^{-10}	15.54	0.6	3.8
GABA	1.9×10^{-10}	15.83	0.6	3.6
Ala	4.5×10^{-10}	16.38	0.6	3.7
Gly	1.9×10^{-10}	16.97	0.7	3.7
Glu	5.8×10^{-10}	20.46	0.8	4.1
Asp	6.6×10^{-10}	21.33	0.8	5.4

were 0.997, 0.997, 0.996, 0.994, 0.999, 0.997, 0.994, respectively.

3.4. Effects of exhausting exercise on amino acids in the extracellular space of hypothalamus

The healthy adult SD male rats with a guide cannula in the awake state before exercise were sampled by microdialysis. The next day, these rats were forced to continuously swim until exhaustion in one test period. The exercised rats were sampled in the awake state at 4 and 72 h after the exercise, respectively. The changes of some amino acids in the hypothalamus extracellular fluid of rats were observed before and after the exercise (Table 2). Table 2 shows that after exhausting exercise, most amino acids from the hypothalamus area were increased markedly. Seventy-two h after the exercise, the concentration of amino acid neurotransmitters were reduced, but still higher than before exercise. The levels of inhibitory amino acid neurotransmitters (e.g. GABA and gly) were far more than excitatory amino acid neurotransmitter (e.g. glu, asp) in hypothalamus extracellular fluid. In recovering from exercise fatigue, the recovery speed and extent of amino acids in the hypothalamus area of rats are different for different amino acids. These data suggest that the amino acid neurotransmitters in the hypothalamus area may play important roles in the central effects of exercise.

E.A. Newsholme et al. suggested that changes of amino acid concentration in plasma could play a role in central fatigue by increasing the rate of synthesis and hence the level of the neurotransmitter 5-hydroxytryptamine (5-HT) in some part of the brain [19]. Aquilo et al. reported that concentrations of most amino acid in blood cells did not change after acute intense exercise. In plasma, glu, arg, ala increased by 29, 13 and 40% respectively, lys decreased by 15%, and asp did not change after acute intense exercise [20]. In the present work, the results showed that changes of amino acid concentration in the hypothalamus extracellular fluid in the brain are far more than the amino acid concentration in plasma after acute intense exercise. Glu is an excitatory amino acid, and plays a central metabolic role in the brain. It is released from neurons in response to neuronal stimulation and is subsequently taken up by both neurons and glia [21]. Glu has difficultly entering into the brain from plasma across the blood–brain barrier [22], and is a precursor of the inhibitory neurotransmitter *r*-aminobutyric acid too [23]. The result of our study showed that the release of glu in the hypothalamus is notably increased after exercise. Whereas 72 h after the exercise, concentration of glu was not different from before exercise. Thus, it is possible that change of glutamate concentration in the brain may play an important role in central fatigue. In this work, the increase of asp was not of statistical significance. Seventy-two h after exercise, the concentration of inhibitory amino acid neurotransmitters (gly, GABA) is still higher than before exercise. The experimental results supported the mechanism of central exercise fatigue–protective inhibition theory. The increase of arg, ala and lys might be due to the changes of synthesis and metabolism of proteins in the brain after exhausting exercise. During exercise fatigue, these amino acids in the brain act as a kind of latent energy to maintain brain function activity.

It is established that the insertion of a microdialysis probe into the brain produces a traumatic brain injury [24–27]. Groothuis et al. [28] reported the blood–brain barrier permeability is increased after insertion of the microdialysis probe into the brain, but blood–brain barrier permeability between day 1 and 7 after insertion of the microdialysis probes into the brains showed no significant differences. Lutgers et al. [29] discussed microdialysis measurement of glucose in subcutaneous adipose tissue up to 3 weeks in Type 1 diabetic patients. The results showed that the recovery of glucose and

Table 2
Concentrations of amino acids in microdialysates from hypothalamus of rats before and after exercise ($\times 10^{-6}$ mol/l, $n=4$)

Analytes	Before exercise	4 h post-exercise	72 h post-exercise
Arg	2.88±0.17	6.46±0.41*	3.47±0.37
Lys	11.26±0.27	28.86±1.50*	19.79±2.24*
GABA	2.96±0.10	7.38±0.38*	4.00±0.55*
Ala	5.60±0.31	14.28±0.69*	10.29±1.33*
Gly	6.22±0.51	11.36±0.92*	10.53±1.42*
Glu	1.59±0.08	3.48±0.33*	1.42±0.18
Asp	0.28±0.07	0.33±0.03	0.25±0.05

* indicate $P<0.05$ as compared with before exercise. n indicates number of rats.

variability in measurements between day 4 and 18 after insertion of microdialysis probes into the subcutaneous adipose tissue did not change appreciably. These results suggest the microdialysis probe can be used to monitor low molecular mass substance from a variety of biological systems for weeks.

In summary, we have demonstrated that capillary electrophoresis coupled laser-induced fluorescence is an efficient and sensitive method to perform the analysis of multiple components in complex microdialysates. The present study utilized these techniques to detect the changes of amino acid neurotransmitters from the hypothalamus extracellular fluid of the rats before and after exercise. After exhausting exercise, glu, GABA, gly, arg, ala and lys were increased noticeably. The results suggest that the amino acid neurotransmitters (glu, GABA, gly, asp) in the hypothalamus area may play important roles in the central effects of exercise.

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