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Simultaneous monitoring multiple neurotransmitters and neuromodulators during cerebral ischemia/reperfusion in rats by microdialysis and capillary electrophoresis

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ABSTRACT

In this work, focal cerebral ischemia/reperfusion was induced by the model of middle cerebral artery occlusion. The dialysates of extracellular fluid in hypothalamus were obtained by using brain microdialysis technique. An efficient and sensitive chiral capillary electrophoresis with laser-induced fluorescence detection procedure was developed for the simultaneous determination of multiple amino acids neurotransmitter including Arg, Lys, Trp, GABA, L-Ser, Ala, Tau, Gly, Glu and Asp; catcholamine (dopamine) and neuromodulator (D-Ser and *O*-phosphoethanolamine) in microdialysate. Different parameters that influenced CE separation were optimized. The optimal method was used to investigate the dynamic changes of neurotransmitters and neuromodulators during cerebral ischemia/reperfusion. The results indicated that extracellular levels of multiple neurotransmitters and neuromodulators were elevated during cerebral ischemia/reperfusion. The dynamic changes and functional status of releasable neurotransmitters and neuromodulators during ischemia/reperfusion were discussed.

1. Introduction

Ischemic stroke is the third largest cause of mortality and the most prevalent neurodegenerative disease in adults (Read et al., 1999). In human ischemic stroke, recirculation occurs frequently after focal ischemia, which aggravates neuronal damage (Sinha et al., 2002). There are yet no clinically effective therapeutic protocols for this disease, because the pathophysiology involved therein is complex and not well understood. Middle cerebral artery occlusion followed by reperfusion is a model of focal ischemia in rats, which resembles to that of human ischemic stroke and has been widely used in the pathological researches of ischemic stroke (Longa et al., 1989).

In the nearly two decades since Benveniste et al. (1984) demonstrated ischemia-evoked releases of glutamic acid (Glu), aspartic acid (Asp) in the rat hippocampus, the neurochemical processes during cerebral ischemia/reperfusion (I/R) have been the subjects of numerous studies; several reviews described that development in detail (White et al., 2000; Phillis and O'Regan, 2003). A number of HPLC methods for the separation and quantification of amino acids in microdialysates during I/R have been developed (Uchiyama-Tsuyuki et al., 1994; Lo et al., 1998; Guyot et al., 2001; Pilitsis et al., 2001). In most of them, researches were focused on excitatory neurotransmitters (Glu and Asp) and inhibitory neurotransmitters (γ-amino-n-butyric acid, glycine and taurine); derivatization with *o*-phthalaldehyde (OPA) was carried out. It is well recognized that cerebral ischemia induces the excessive releases of Glu and Asp, which provoke enzymatic process leading to irreversible neuronal injury (Krause et al., 1988; Phillis and O'Regan, 1996; Guyot et al., 2001). However, there are various kinds of neurotransmitter and neuromodulator in brain, the level of neuronal excitability is determined by the relative balance between these neuroactive substances and alteration in such a balance is thought to underlie some neurological disorders (Davies, 1999; Sharma, 2007). It is therefore of particular interest to simultaneously monitor the extracellular levels of multiple neurotransmitters and neuromodulators in brain. Only a few methods, however, describing the simultaneous determination of multiple neurotransmitters and neuromodulators during I/R are well validated. Lo et al. (1998) reported a HPLC method with OPA derivatization for measuring eleven kinds of amino acid during I/R; however, catcholamine transmitter dopamine could not be detected. Hemelrijck et al. (2005) determined eight kinds of amino acid associated with cerebral ischemia in rat brain microdialysates using narrowbore HPLC with OPA derivatization. Fraser et al. (2008) developed a HPLC method with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

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derivatization for determining eleven kinds of amino acid during ischemia in preterm fetal sheep. However, neuromodulator D-serine (D-Ser) and *O*-phosphoethanolamine could not be analyzed in the above two reports. Moreover, the disadvantage of these HPLC methods is the rather long analysis time (50–100 min), which makes it difficult to keep up with the microdialysis sampling frequency. Till now, the dynamic changes of multiple neurotransmitters and neuromodulators during cerebral I/R have not been extensively investigated, which is worth investigating for understanding the pathological processes in ischemic stroke.

In the recent years, since new roles have been proposed for neuromodulator D-Ser in stroke (Hamase et al., 2002), it is particularly meaningful to quantify it. D-Ser has been identified as an endogenous ligand for the glycine site of NMDA receptor, which plays important functions in physiological and pathological processes (Wolosker et al., 2002; Mothet et al., 2000; Katsuki et al., 2004). Hashimoto et al. (1992a,b) reported the first detection of D-Ser in rat tissues by using GC–MS and HPLC methods. Since then, several improved methods have been developed, most of which involve precolumn chiral derivatization of amino acids and subsequent HPLC separation (Lo et al., 1998; Morikawa et al., 2001). However, this conventional method suffers from high consumption of expensive chiral derivatization reagents and samples. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)-based procedure was reported for the separation of D/L-Ser enantiomers (Zhao et al., 2005; Thongkhao-On et al., 2004; Ciriacks and Bowser, 2006). Proper selection of a labeling reagent requires weighing the needs for detection sensitivity, derivative stability and cost. In this work, precolumn fluoresceine-5-isothiocyanate (FITC) labeling was selected because of its benefits in high quantum yield, stable derivatives and low price. Moreover, the excitation/emission wavelengths of FITC are 490/518 nm, so the commercially available argon-ion laser can be used.

Although the changes in levels of several extracellular amino acids were examined in various models of global and focal cerebral ischemia (Lo et al., 1998; Phillis and O'Regan, 2003; Hemelrijck et al., 2005), there are apparently no reports available about the dynamics of multiple neurotransmitters and neuromodulators including D-Ser in the model of middle cerebral artery occlusion (MCAO).

The hypothalamus is one of the important central for the regulation of autonomic, endocrine and energy homeostasis. There are multiple neurotransmitters and neuromodulators in hypothalamus (Brooks, 1988). Investigation of the extracellular levels of neurotransmitters and neuromodulators in hypothalamus provides valuable insights into the pathophysiological events that occur during I/R. In this study, the dialysates of extracellular fluid in hypothalamus were obtained by brain microdialysis technique. Focal cerebral ischemia/reperfusion was induced by the model of MCAO. We sought to establish an efficient and sensitive chiral MEKC analytical method for the simultaneous determination of a wider spectrum of neurotransmitters and neuromodulators including D-Ser in microdialysate, by which the dynamic changes of extracellular amino acids and amines during cerebral I/R were investigated.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. L-Arginine (Arg), L-lysine (Lys), L-methionine (Met), L-proline (Pro), L-glutamine (Gln), L-histidine (His), L-leucine (Leu), L-isoleucine (Ile), L-phenylalanine (Phe), L-tryptophan (Trp), γ-amino-n-butyric acid (GABA), D-serine (D-Ser), L-serine (L-Ser), L-taurine (Tau), L-alanine (Ala), L-glycine (Gly), L-glutamic acid (Glu), L-aspartic acid (Asp) were obtained from Shanghai Reagent Corp. (Shanghai, China). 2,6-O-Dimethyl-β-cyclodextrin (DM-β-CD) was purchased from Beijing Chemical Industry University (Beijing, China). 2,3,5-Triphenyltetrazolium chloride (TTC), 2-hydroxyproxypropyl-β-cyclodextrin (HP-β-CD), fluorescein isothiocyanate isomer I (FITC), *O*-phosphoethanolamine (PEA), dopamine (DA) and SDS were purchased from Sigma (St. Louis, MO, USA). FITC stock solution (1 mM) was prepared in acetone and stored at 4 °C in the dark. The stock solutions of amino acids (0.1 mM) were prepared in deionized water and diluted to the desired concentration prior to use. A stock solution of DA (0.1 mM) was prepared by dissolving DA in 0.3% (v/v) acetic acid (5:1, v/v) and diluted to the desired concentration with acetic acid (5:1, v/v). Water was purified by Millipore-Q system (Millipore, USA) and used for all solutions.

2.2. CE apparatus and operation conditions

All CE experiments were performed on a Beckman MDQ Capillary Electrophoresis System equipped with Beckman Laser Module 488 nm Argon-ion laser (Beckman-Fullerton CA, USA). Uncoated fused-silica capillaries of 57 cm (50 cm to detector) × 75 μm I.D. (Yongnian Optic Fiber Inc., Hebei, and China) were used in the CE procedures. New capillaries were pre-treated with 1 M NaOH for 60 min followed by water for 60 min at room temperature. Prior to use, the capillaries were sequentially flushed with 0.1 M NaOH, ultrapure water for 2 min and then running buffer for 3 min. The above flushing cycle was repeated to ensure the separation reproducibility for each injection. Sample introduction was performed by hydrodynamic injection at 0.5 p.s.i. for 5 s. The typical CE separation conditions were a background electrolyte consisting of 15 mM borate buffer (pH 10.2) with 70 mM SDS, 5% methanol, 17.5 mM HP-β-CD and 5 mM DM-β-CD, with a voltage of 22.5 kV and a temperature of 25 °C.

2.3. Precolumn derivatization of amino acid standards and microdialysis samples

Precolumn derivatization of standard amino acids and amines were conducted in microvials. After appropriate amounts of standard solutions were diluted and mixed in 5 mM borate buffer (pH 9.6), FITC was added to given a final concentration 30 times higher than the total concentration of amino acids and amines. Typically, the derivatization reactions were performed at room temperature (20 °C) in the dark for 16 h. The derivatization solutions were then stored in the dark at −20 °C before use.

Aliquots of 6 μl of microdialysates, 10 μl of 5 mM borate buffer (pH 9.6) and 4 μl of FITC stock solution (1 mM) were added sequentially to microvials. The derivatization reactions were performed and reacted at room temperature (20 °C) in the dark for 16 h. Derivatization solutions were diluted 3 times with the running buffer before CE analysis.

2.4. Cerebral ischemia/reperfusion injury model and microdialysis experiment

2.4.1. Animals, stereotaxic surgery and microdialysis procedure

All animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University and were in accordance with the "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1996). The Male Sprague–Dawley rats (three months of age) were purchased from Hubei Academy of Medical Science (Wuhan, China). The rats were housed individually with food and water available ad libitum. Foods were withdrawn from all rats 12 h before surgery. Body temperature was maintained between 36.5 °C and 37 °C with a heating pad during