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Monitoring of extracellular pyruvate, lactate, and ascorbic acid during cerebral ischemia: a microdialysis study in awake gerbils

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Abstract

In vivo microdialysis coupled with liquid chromatography was developed for the continuous monitoring of brain neurochemicals during cerebral ischemia in awake, free moving gerbils. The dead volume of the microdialysis system was estimated to be less than 30 μl. The detection limits of the present assay were 0.2 to 2.0 μ*M* for analytes at a signal to noise ratio of five. To validate this assay, a focal cerebral ischemia was produced by occlusion of one common carotid artery for 60 min and then reperfusion for additional 3 h in awake gerbils. A microdialysis probe was inserted into the striatum of the gerbil. Dialysates were autoinjected and analyzed extracellular pyruvate, lactate, and ascorbic acid by liquid chromatography with a UV detector during cerebral ischemia. Significant changes of pyruvate and the lactate/pyruvate ratio were observed. Biphasic and dynamic changes in ascorbic acid and lactate were proposed to correlate a secondary damage. This assay can be used as a tool to study dynamic changes of brain neurochemicals in awake animals.

Keywords: Microdialysis; Gerbils; Pyruvate; Lactate; Ascorbic acid

1. Introduction

Cerebral ischemia results in low oxygen and glucose supply and causes decreased adenosine triphosphate (ATP) formation [1,2]. Various ATP-driven membrane-bound pumps or reuptake processes that usually work in the homeostasis of important metabolites or ions become retarded during cerebral ischemia. Moderate to severe neuronal damages might occur following these events. In addition, secondary neuronal injury might occur, becoming worse during reperfusion or after reperfusion when the above pumps or reuptake channels resume. The damage of central enzymes seen after a period of ischemia by reperfusion has been termed ''secondary ischemia''. The selective vulnerability of certain neurons to cerebral ischemia is well established morphologically [3–5]. It has been attributed to various biochemical processes. Among them, glutamate and some cellular energy related metabolites, such as pyruvate, lactate, play important roles in the secondary ischemia [6–8].

Microdialysis was introduced two decades ago for sampling neurochemical substances from the extracellular fluid of the brain [9–11]. These neurochemical changes of brain can be measured over time. On-line microdialysis provides for automatic

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sampling and the acquisition of dynamic information of the brain, such as how events or processes are reflected in the local compartments [12,13]. Microdialysis studies on cerebral ischemic processes have mainly been based on anesthetized animals [14–16]. Findings obtained by anesthetized animals may involved some mechanisms which are dependent on mediated transmission. Anesthesia agents may also modulate the activity of neurotransmitters.

Brain lactate and pyruvate levels as well as the lactate/pyruvate ratio have been advocated for estimation of the severity of stroke and also as tools for prognostication of the outcome [17,18]. Lactate is produced by nerve tissues, including nervous and glial cells under cerebral ischemic conditions [17–19]. Cerebral ischemia might damage the integrity of cellular membrane of brain tissues, and thus, intracellular lactate and ascorbic acid are released into the extracellular space [19]. Ascorbic acid release may also be regulated by glutamate transports which are altered during cerebral ischemia. Under anaerobic conditions, pyruvate is reduced to lactate catalyzed by lactate dehydrogenase. Changes in concentration of lactate, pyruvate, ascorbic acid, and the lactate/pyruvate ratio may serve as biochemical markers of impending cerebral ischemia.

It seems reasonable to expect that ischemic mediators may dynamically change in varied stages of cerebral ischemia. Previous studies have not concentrated on the nature of the earliest ischemia and reperfusion-related changes in concentration of pyruvate, lactate, ascorbic acid, and the lactate/pyruvate ratio. Understanding these initial changes and reperfusion event may make it easier to explore the primary cellular events that eventually lead to ischemic injury or delayed neuronal death. The simultaneous determination of pyruvate, lactate, and ascorbic acid is important for the investigation of the brain injury mechanism in earliest ischemia and reperfusion [17–19]. The aim of the present study was to investigate energy related metabolite changes in an unilateral cerebral ischemic event of gerbils via a rapid on-line microdialysis hyphenated technique.

2. Experimental

Standard stock solutions of pyruvate and L-lactate were prepared at concentrations of 10 and 100 m*M*, respectively, in 4 m*M* sulfuric acid and stored at 4°C. The standard mixtures were prepared from a portion of these stock solutions after appropriate dilution with 4 m*M* sulfuric acid. Ascorbic acid was prepared fresh daily at a concentration of 100 μ*M*. In vitro recovery was also performed in a standard mixture containing pyruvate, lactate and ascorbic acid, to determine the recoveries of all analytes and validate each probe prior to experiments. In the meantime, the dead volume of the microdialysis system was also determined.

Gerbils were anesthetized with ketamine (20 mg/kg) administered intraperitoneally, and body temperature was maintained at 37°C with a heating pad (CMA/150). The gerbil's head was mounted on a stereotaxic apparatus (Davis Kopf Instruments Inc., Tujunga, CA) with the nose bar positioned 4.0 mm below the horizontal line. Following a midline incision, the skull was exposed and one burr hole was made on the skull for the insertion of a guide cannula (CMA/12, Carnegie Medicin, Stockholm, Sweden). The guide cannula was lowered at the top of striatum (AP 0 mm, ML −3.5 mm to bregma, DV −2.0 mm from dura) and secured to the skull using dental cement with two skull screws. After surgery gerbils were allowed to recover for 12–24 h prior to experiments.

On the day of experiment, each gerbil was habituated to the dialysis chamber. Prior to the probe insertion, the dialysis probe was perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{++}; 4 m*M* K^+; pH 7.0) at a flow-rate of 3 μl/min. The microdialysis probe (CMA/12, 4 mm in length) was then inserted into the striatum. Dialysates were collected every 10 min in a CMA/160 autoinjector. Dialysates (5 μl) were directly injected onto an LC system with a UV detector set at 214 nm (BAS UV-116A, Bioanalytical Systems, West Lafayette, IN) for the determination of pyruvate, lactate, and ascorbic acid. Separation of these substances was achieved using a conventional Polypore column (220×4.6 mm I.D.) packed with 10 μm Polypore H (Brownlee Lab, Deerfield, IL). Following the insertion of the dialysis probe and a 2 h equilibration period, baseline samples were collected and a stable baseline was achieved. Gerbils were anesthetized with chloralhydrate (360 mg/kg, i.p.). The right

common carotid artery, exposed through a ventral midline incision in the neck, was carefully separated from the vago-sympathetic trunks and was occluded for 60 min with an aneurysm clip (Aesculap AG, Tuttlingen, Germany) producing a unilateral cerebral ischemia. Then, reperfusion was performed for an additional 3 h.

The mobile phase consisted of 4 m*M* sulfuric acid in doubled distilled water (112 μl concentrated sulfuric acid in 1000 ml distilled water). The mixture was filtered through a 0.22 μm Nylon filter under reduced pressure and degassed by helium for 20 min. The flow-rate was 0.5 ml/min, at a maintained column pressure of ca. 5.7 MPa. The identity of each peak on the chromatogram was confirmed by it retention time and a superimposed technique which was provided by HP (Hewlett Packard 3365 Series II ChemStation, Taiwan Branch, Taipei, Taiwan). Concentrations of pyruvate, lactate, and ascorbic acid in dialysates were calculated according to calibration curves.

3. Results and discussion

Fig. 1A shows a typical chromatogram of in vitro recovery of a microdialysis probe in a standard mixture containing 0.05, 0.05, and 0.5 m*M* pyruvate, ascorbic acid, and lactate. Analysis was completed within 10 min. The dead volume of this microdialysis system was estimated to be less than 30 μl. Therefore, the representative analysis of an event was delayed by approximately 10 min (or one interval).

Calibration curves were constructed with three standard mixtures each of pyruvate (2, 50, and 100 μ*M*), ascorbic acid (10, 50, and 100 μ*M*), and lactate (20, 500, and 1000 μ*M*), prior to LC analysis of microdialysates. The amounts of each injected analyte were linearly related to chromatographic area obtained from standard mixtures. In general, correlations (R^2) for pyruvate, ascorbic acid, and lactate on typical calibration curves, were linear ($R^2 \geq 0.999$).

The precision and stability of the assays were tested using a standard mixture containing 100 μ*M* pyruvate, 100 μ*M* ascorbic acid, and 1000 μ*M* lactate in 0.1 *M* HCl and pooled microdialysates. The intra-assay variabilities were assessed with 12 replicates at 1 h intervals and expressed as coefficients of variation (C.V., %). In general, C.V. values for pyruvate, ascorbic acid, and lactate in various matrixes and the pooled dialysate were acceptable (<3.0%) in the intra-assay variability. The inter-assay variabilities were assessed with standard mixtures containing 100 μ*M* pyruvate, 100 μ*M* ascorbic acid, and 1000 μ*M* lactate in 0.1 *M* HCl over five consecutive working days. C.V. values for pyruvate and lactate were much lower (3.1 and 3.4%) than that for ascorbic acid (7.3%) in the chromatographic measurements. Ascorbic acid solution was prepared daily by weighing a small amount of the drug, leading to a relatively higher C.V. value, whereas pyruvate and lactate could be prepared from a stock solution. The detection limits (signal-to-noise ratio=5) of all analytes in the present assay were between 0.2–2.0 μ*M* (0.2 μ*M* for pyruvate, 0.5 μ*M* for ascorbic acid, and 2.0 μ*M* for lactate).

In awake gerbils, 2 h after insertion of the probe, basal concentrations ($n=4$) of all analytes in striatal dialysates were obtained. Fig. 1B showed a typical baseline chromatogram of a microdialysate obtained from a gerbil striatum prior to the ligation. The retention times of each peak corresponding to pyruvate, ascorbic acid, and lactate in Fig. 1B were identical to those in Fig. 1A. All studied analytes were well resolved. The basal concentrations of pyruvate, ascorbic acid, and lactate were 11.5, 7.2, and 87.8 μ*M*, respectively. Basal pyruvate and ascorbic acid levels were about twice when compared to that of anesthetized gerbils in our previous study [16]. The variations could simply represent different degree of utilization of glucose in anesthetized and awake animals. The measurements of pyruvate, ascorbic acid, and lactate in such small volumes and with such low detection limits in awake animals are of great analytical potentials in microdialysis experiments. Ligation of the right common carotid artery producing a unilateral cerebral ischemia was performed after obtaining basal levels of all analytes. A typical chromatogram of a microdialysate obtained from a gerbil striatum at 30 min after ligation was shown in Fig. 1C. Significantly increased lactate level and decreased pyruvate level were observed.

The entire time profiles of extracellular pyruvate, lactate, ascorbic acid, and the lactate/pyruvate ratio

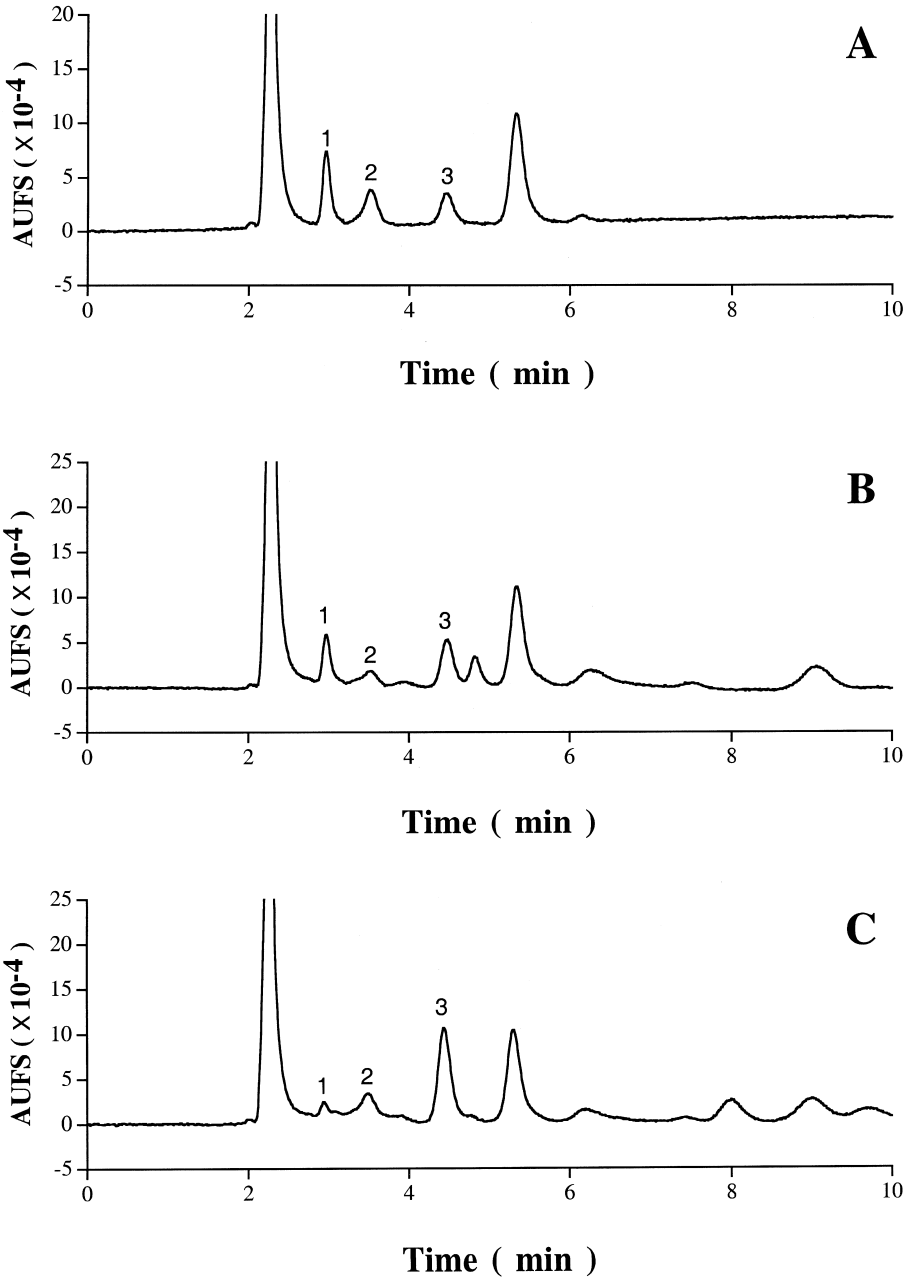

Fig. 1. Typical chromatograms obtained from (A) in vitro recovery of a standard mixture containing (1) pyruvate, (2) ascorbic acid and (3) lactate; (B) a baseline striatal microdialysate obtained prior to a unilateral ligation; (C) a striatal microdialysate obtained at 30 min after ligation. (AUFS: absorbance unit full scale.)

in the striatum before and after a 60 min unilateral cerebral ischemia were constructed. It is evident that these analytes varied very soon after cerebral ischemia and reperfusion. Extracellular pyruvate level drastically decreased to 23% of the baseline during the 60 min ligation of the right common carotid

artery. It gradually returned to the baseline within 2 h and slightly decreased at the end of reperfusion stage.

Lactate gradually increased to a plateau of 419% of baseline level in the first 30 min and then gradually decreased to 245% of the baseline level during the ligation period. It further increased to 857% of baseline level during the first 1 h reperfusion and slowly decreased to about 360% within 3 h reperfusion. The mechanism by which ischemia causes increased lactate levels in the extracellular space probably reflects both an increased release of lactate into the extracellular space as well as a decreased removal of lactate from the extracellular space. Removal of lactate from the extracellular space is largely due to an uptake mechanism which is an adenosine triphosphate (ATP)-dependent process. Ischemia is known to cause ATP depletion within 5 min. Therefore, lactate uptake mechanism is blocked and lactate levels accumulate.

The basal lactate/pyruvate ratio was about 8.1 prior to the ligation. The ratio increased significantly to 128 at the end of the unilateral ligation. The ratio gradually decreased to about 41 at 60 min following reperfusion. In general, increased lactate levels and decreased pyruvate production by tissues were demonstrated during cerebral ischemia, simply because of a decreased regional cerebral blood flow.

Ascorbic acid also demonstrated a biphasic pattern of increase in the ligation period and latter in the reperfusion period. Our data indicated biphasic increases in lactate and ascorbic acid levels during cerebral ischemia/reperfusion of awake gerbils. The early phase of increasing these metabolites could be stimulated by a decreased cerebral blood flow, whereas the second phase of increasing these metabolites could indicate a secondary injury caused by reperfusion. These data are in agreement with those reported by other investigators [20]. The detailed mechanism of the biphasic increases in ascorbic acid and lactate levels during unilateral cerebral ischemia requires further investigation.

The gerbil brain lacks the connection between the carotid and vertebrobasilar circulation, which makes the circle of Willis incomplete [21]. Little anteroposterior blood flow exists in the gerbil brain. Therefore, a right common carotid artery ligation will prevent blood flow to anterior portion of the right brain. This cerebrovascular plan has made the gerbil brain an excellent model for studying cerebral ischemia. These chromatograms and data clearly demonstrate gerbil striatal extracellular energy-related metabolites and an antioxidant, ascorbic acid, in a near ''real time'' manner. The studies yielded the entire time profiles of dynamic information on chemical changes in awake gerbils subjected to unilateral cerebral ischemia.

4. Conclusion

The monitoring energy related metabolites in the extracellular fluid with microdialysis could provide useful information on energy metabolic disturbance with high temporal and spatial resolutions. An on-line microdialysis coupled with liquid chromatography was developed for the continuously monitoring of striatal pyruvate, lactate, and ascorbic acid levels. The detection limits of the present assay, at a signal-to-noise ratio of five, were 2.0, 0.2 and 0.5 μM, for lactate, pyruvate, and ascorbic acid, respectively. Significant and dynamic changes in pyruvate and the lactate/pyruvate ratio were observed in awake, free moving gerbils subjected to a 60 min unilateral ischemia. Biphasic increases of ascorbic acid and lactate during cerebral ischemia/reperfusion were clearly demonstrated. The use of an on-line microdialysis hyphenated technique provides a near ''real-time'' monitoring of chemical changes with a great potential for investigating detailed intracranial dynamics. An assay by which the energy states of the brain can be monitored is valuable in a number of acute experimental animal models and clinical situations. Furthermore, recent research has pointed out several cerebral ischemic processes leading to delayed neuronal cell death, such as lactateosis. A number of anti-ischemic drugs are currently being evaluated in cerebral ischemic animal models and clinical trials.

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