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Simultaneous monitoring of extracellular glucose, pyruvate, lactate and glutamate in gerbil cortex during focal cerebral ischemia by dual probe microdialysis

Dar-Yu Yang[a,c], Tung-Hu Tsai[b], Chi-Hung Cheng[d], Chi-Wen Lee[e], Sy-Huang Chen[e], Fu-Chou Cheng[e,*]

[a]*Department of Emergency, Chung-Shan Medical and Dental College, Taichung 402, Taiwan*
[b]*Department of Pharmacology, National Institute of Chinese Medicine, Taipei 112, Taiwan*
[c]*Department of Emergency, Taichung Veterans General Hospital, Taichung 407, Taiwan*
[d]*Section of Nephrology, Taichung Veterans General Hospital, Taichung 407, Taiwan*
[e]*Department of Medical Research, Taichung Veterans General Hospital, Taichung 407, Taiwan*

Abstract

The aim of this study was to monitor dynamic changes in energy-related metabolites in the cortex of gerbils subjected to cerebral ischemia by a dual probe microdialysis technique. Focal cerebral ischemia was produced in anesthetized gerbils by occlusion of the right common carotid artery and the right middle cerebral artery for 60 min. Two microdialysis probes were inserted into both sides of the cortex to simultaneously monitor extracellular glucose, lactate, pyruvate and glutamate. Dynamic and comparative changes in these analytes, on the ipsilateral and contralateral sides of the brain, were simultaneously monitored by liquid chromatography and a microdialysis analyzer. The present study demonstrated decreases in glucose and pyruvate, increases in lactate and glutamate on the ipsilateral side whereas all analytes remain constant on the contralateral side of cortex during cerebral ischemia. In vitro recovery of each microdialysis probe was performed to ensure the quality of experiments. The detection limits of pyruvate, glutamate, lactate and glucose were 0.2, 1.0, 2.0 and 20 μM, respectively. The intra- and inter-assay correlations were less than 5% in standard mixtures and pooled brain dialysates.

Keywords: Microdialysis; Glucose; Pyruvate; Lactate; Glutamate; Organic acids

1. Introduction

Microdialysis was introduced 2 decades ago and is widely used for sampling of neurochemical substances from the extracellular fluid of the brain [1–3]. Today it is one of the most widely used techniques for in vivo sampling of the chemical environment of the brain. Carotid occlusion in Mongolian gerbils has been employed extensively as a model of cerebral ischemia analogous to some forms of human stroke [4–7]. Isolated cerebral hemispheres and incomplete cerebral circle of Willis are unique anatomic features of gerbils [8,9]. Each brain hemisphere seems to have an independent blood supply, thus two microdialysis probes can be used to simultaneously monitor dynamic changes in neurochemicals on ipsilateral and contralateral sides of the brain during cerebral ischemic event [10].

*Corresponding author. Tel.: +886-4-3592-525; fax: +886-4-3592-705.
E-mail address: fucheng@vghtc.vghtc.gov.tw (F.-C. Cheng).

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Metabolic changes on the ipsilateral side can be compared with those on the contralateral side, which being almost intact can serve as a control in gerbils.

Cerebral ischemia results in low oxygen and glucose supply and causes decreased ATP formation [11–13]. Various ATP-driven membrane-bound pumps or reuptake processes that usually work to keep the homeostasis of important metabolites or ions become retarded. Moderate to severe neuronal damage might occur following ischemic events. Large amounts of lactate can be produced by nerve tissues, including nerve and glial cells, in acute cerebral ischemia. Under anaerobic conditions, pyruvate is reduced to lactate by lactate dehydrogenase. Lactate levels in brain have been advocated for estimating the severity of stroke and for prognostication of the outcome [14,15]. Changes in glucose, lactate, pyruvate and glutamate may serve as important biochemical markers of cerebral ischemia in experimental animals and clinical studies [14,15].

The immunosuppressant FK506 has recently been introduced into clinical use for the prevention of allograft rejection [16]. In addition to its immunosuppressive properties, it has powerful neuroprotective effects in animal models of focal cerebral ischemia [17,18]. FK506 inhibits calcineurin, the only known protein phosphotase that is under the direct control of intracellular calcium. Calcineurin can be activated by elevation of intracellular calcium levels under ischemic conditions [18]. However, in neuronal tissues, extracellular changes caused by ischemic insult following treatment with FK506 have not been investigated. In the present study, dynamic changes in energy related metabolites in cortex were investigated by a dual probe microdialysis hyphenated technique during focal ischemia following pretreatment with FK506.

2. Materials and methods

Standard stock solutions of glucose, pyruvate, lactate and glutamate were prepared at concentrations of 1–10 m*M* in double distilled water and stored at 4°C. A standard mixture was prepared from a portion of stock solutions after appropriate dilution. All reagents were of analytical quality unless otherwise stated.

Six male gerbils (65–75 g) were randomly assigned to the control and FK506 groups, and received injection (intraperitoneal, i.p.) of saline or FK506 (1 mg/kg) 30 min prior to ligation. The animals were anesthetized with chloral hydrate (360 mg/kg, i.p.) and body temperature was maintained at 37°C with a heating pad (CMA/150). The right common carotid artery (CCA), exposed through a ventral midline incision in the neck, was carefully separated from the vago-sympathetic trunks and loosely encircled with sutures for later occlusion. The gerbil's head was mounted on a stereotaxic apparatus (Stoelting, IL, USA) with the nose bar positioned 3.3 mm below the horizontal line. Following a midline incision, the skull was craniectomized to expose the right middle cerebral artery (MCA). An 8-0 suture (blue monofilament polypropylene, DG, Davis-GECK, Wayne, NJ, USA) was positioned so that it encircled the middle cerebral artery for later ligation. Two microdialysis probes (polycarbonate membrane 4 mm in length, with molecular mass cut-off 20 000, CMA/12, Carnegie Medicin, Stockholm, Sweden) were stereotaxically implanted into the cortex (AP 0 mm, ML ±5 mm, DV −4.0 mm from bregma). A focal ischemic/reperfusion lesion was made by simultaneous occlusion of the right CCA and the right MCA for 60 min followed by 3 h reperfusion.

Generally, there were two difficulties in performing microdialysis and the simultaneous occlusion of the MCA and CCA. The tiny diameter and the location of MCA was difficult to approach in small animals, such as gerbils and mice. Careful craniectomy was done to expose MCA for encircling the MCA by an 8-0 suture. In addition, There is almost no space to work (occlusion of a common carotid artery) below the neck of gerbil when the gerbil is immobilized on a stereotaxic frame. The use of a micro aneurysm (FE717K) for the ligation of CCA via a 90° angled Caspar Applying Forcep (FE-526K. Aesculap, Tuttlingen, Germany) could solve this problem.

A laser probe (0.8 mm in diameter) of a laser doppler blood flow monitor (MBF 3D, Moor Instruments, Axminster, UK) was positioned in the cortex with its tip close to the middle cerebral artery. The flow signal was averaged with a 5-s time constant, and recorded continuously on an *x*–*y* recorder.

Dialysis probes were perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+; pH adjusted to 7.0) at 2 μl/min using a CMA/100 microinfusion pump. Dialysates were collected every 15 min in a CMA/140 fraction collector (Carnegie Medicin). Aliquots of dialysates (5 μl) were injected onto a liquid chromatography (LC) system with a UV detector (BAS UV-116A, Bioanalytical Systems, West Lafeyette, IN, USA) for the determinations of lactate and pyruvate [19]. Separation of these substances was achieved using a conventional Polypore H column (100×4.6 mm I.D., 10 μm, Brownlee Lab., IL, USA). The mobile phase consisted of 4 m*M* sulfonic acid in double-distilled water. The mobile phase was filtered through a 0.22-μm nylon filter under reduced pressure and degassed with helium for 20 min. The flow-rate was 0.5 ml/min with a maintained column pressure of ca. 5.2 MPa. The concentrations of pyruvate and lactate in dialysates were evaluated by determining each peak area ratio relative to the standard mixture. The identities of peaks on chromatogram were confirmed by retention times and a superimposing technique provided by Hewlett-Packard (Hewlett-Packard 3365 Series II ChemStation, Taiwan Branch, Taipei, Taiwan).

Additional aliquots of dialysates (0.5 μl) were assayed in a microdialysis analyzer (CMA/600, Carnegie Medicin) for the determinations of glucose and glutamate. Glucose is oxidized by glucose oxidase in the analyzer. Peroxidase catalyzes the reaction between the hydrogen peroxide formed, phenol and 4-amino-antipyrine to form the red–violet colored quinoneimine. Glutamate is enzymatically oxidized by glutamate oxidase in the analyzer. Peroxidase catalyzes the reaction between the formed hydrogen peroxide, 4-amino-antipyrine and *N*-thyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS) to form the red–violet quinonediimine. The rate of formation of the colored substance is measured at 546 nm and was proportional to the glucose or glutamate concentration, respectively.

3. Results and discussion

In general, calibration curves were obtained with six standard mixtures each of pyruvate (2, 20, 50, 100, 200 and 500 μ*M*) and lactate (20, 200, 500, 1000, 2000 and 5000 μ*M*) prior to experiments. The amounts of each injected analyte were linearly related to chromatographic area obtained from standard mixtures over a large range of concentrations. The correlations (R^2) for pyruvate and lactate on typical calibration curves, were linear ($R^2=1.000$). The detection limits (signal-to-noise ratio=5) for pyruvate and lactate in the present assay were 0.2 and 2.0 μ*M*, respectively. Calibration curves of glucose and glutamate were obtained by the CMA/600 microdialysis analyzer. The deviations of calibration curves ($R^2=0.999$) for glucose and glutamate were less than 5%. The linear ranges of glucose and glutamate were 20–6000 and 1–150 μ*M*, respectively. The detection limits for glucose and glutamate were 20 and 1.0 μ*M*, respectively.

In vitro recovery test was performed in a standard mixture to determine the recoveries of all analytes at 2 μl/min. The in vitro recoveries of a standard mixture containing 10, 10, 100 and 1000 μ*M* pyruvate, glutamate, lactate and glucose were 29±2, 27±2, 25±2, and 24±1%, respectively ($n=10$). Microdialysis probes with appropriate recoveries (>20%) were used to ensure the analytical quality. In addition, each probe was used for no more than four animals to ensure its performance. The dead volume of the microdialysis system was also determined (ca. 10 μl). Therefore, operating procedures have a time lag of 5 min ahead of collecting samples according to the dead volume.

The precision of the assays was tested using a standard mixture and a pooled dialysate sample. The intra- ($n=12$) and inter-assay ($n=6$) correlations were assessed and expressed as relative standard deviations (RSDs), as shown in Table 1. The RSD values for determination of glucose, lactate, pyruvate and glutamate were less than 4.1% in standard mixtures and pooled brain dialysates. The inter-assay variabilities of assessments of with glucose, pyruvate, lactate and glutamate over 6 consecutive days were less than 4.7%. The measurements of glucose, pyruvate, lactate and glutamate achieved with such precision, low detection limits and small volumes demonstrate the analytical potential of microdialysis–LC techniques.

Cerebral blood flow (100% at basal) dropped to 60% of basal when CCA was occluded and then to less than 5% of basal after occlusion of MCA.

Table 1
Analytical precision (RSD, %) on intra-assay (n=12, at 1-h intervals) and inter-assay (n=6, on 6 consecutive working days) stabilities of standard mixtures and brain dialysates in the CMA/600 and the LC–UV system

	RSD (%)			
	Glucose	Pyruvate	Lactate	Glutamate
Intra-assay				
Standard mixture	2.3	3.0	1.5	2.1
Pooled dialysates	1.8	3.5	2.1	4.1
Inter-assay				
Standard mixture	3.1	4.7	2.8	4.6

Cerebral blood flow reached minimal levels within 5 min of the start of occlusion (CCA+MCA) and maintained these levels throughout the occlusion period. The cerebral blood flow gradually returned to basal level (about 80–90%) 10 min after the start of reperfusion and was maintained throughout the experimental period (3 h). Occlusion of the right CCA and MCA prevented cerebral blood flow to anterior portion of the right brain, because the gerbil brain lacks the connection between the carotid and vertebrobasilar circulation, resulting in an incomplete circle of Willis. Thus, unilateral CCA+MCA occlusion in gerbils provides an excellent animal model for studying focal cerebral ischemia.

In general, stable basal levels of analytes in cortex were obtained 2 h after implantation of microdialysis probes. Basal concentrations of glucose, pyruvate, lactate and glutamate in dialysates obtained from both sides of cortex are shown in Table 2. There were no significant differences of basal concentrations of analytes between the two groups except in pyruvate levels. Pyruvate levels were slightly higher in the FK506 group than in the control group. These variations could be attributed to the injection of FK506 30 min prior to ligation.

Glucose levels drastically decreased (5% of baseline) on the ipsilateral side during unilateral ligation of CCA and MCA for 60 min ligation. These levels gradually returned to about 46% of baseline within 3 h reperfusion in the control group. The glucose profile of the FK506 group was similar to that of the control group during the ligation period whereas glucose levels returned to 97% of the baseline within 3 h reperfusion. Using dual probe microdialysis, the results obtained on the ipsilateral and contralateral sides can be compared and contrasted (all the data are based on the assumption that the neurochemicals were at equilibrium before the ligation). On the contralateral side in both groups, glucose levels increased gradually from the beginning of ligation throughout the end of reperfusion (157–196%). These profiles indicate that focal ischemia also affected energy metabolism on the contralateral side of the brain.

Pyruvate, which is an intermediate of glucose metabolism, significantly decreased unilateral ligation of CCA and MCA for 60 min. On the ipsilateral side of the control and FK506 groups, pyruvate levels decreased to 24% and 48% during ligation, respectively. These levels gradually returned to 60% of baseline within the 3 h reperfusion period in the control group. However, pyruvate levels increased to 245% of baseline within 2 h reperfusion period in the FK506 group, then, declined to 123% at the end of experiment. The consumption of pyruvate might be related to the blockade of some transports due to lack of oxygen and glucose during the ischemic event [20]. On the contralateral side, pyruvate levels

Table 2
Baseline concentrations of glucose, pyruvate, and lactate and glutamate on the ipsilateral and contralateral sides were determined using dual probe microdialysis–LC–UV and CMA/600 in the brain of gerbils prior to focal cerebral ischemia with saline or FK506 (n=3) pretreatment

	Concentration (μM)			
	Lipsilateral		Contralateral	
	Saline	FK506	Saline	FK506
Glucose	699.1±141.2	568.8±235.6	622.8±174.5	418.4±180.6
Pyruvate	5.4±1.3	8.7±3.2	3.9±1.6	9.4±3.8
Lactate	339.1±121.4	485.1±165.0	410.3±148.9	483.8±189.3
Glutamate	2.6±1.9	2.3±2.1	3.7±2.9	3.4±3.4

increased during the ligation period, up to 195–197% for both groups. However, these levels gradually declined throughout the reperfusion period. Preserved and enhanced pyruvate levels in the contralateral brain of the FK506 group during reperfusion might be related to the neuroprotective effects of FK506.

The lactate levels in the ipsilateral brain increased and peaked at about 195% of baseline during ligation. Then, lactate levels slightly declined and maintained throughout the reperfusion period in the control group. However, lactate levels showed notable increase (328% of the baseline) during the ligation period in the ipsilateral brain of the FK506 group. Lactate levels further increased to 538% of basal within 1 h after reperfusion and then decreased to 361% of baseline at the end of reperfusion in the FK506 group. On the contralateral sides, moderate increases in lactate levels were observed during the ligation period. Lactate levels gradually declined (down to 40–61%) throughout the end of reperfusion in both groups. The mechanism of significantly enhanced lactate levels in the ipsilateral brain during cerebral ischemia in the FK506 group is unknown.

There was a transient, marked increase in extracellular glutamate in the ipsilateral brains during the ligation period whereas glutamate levels remained at basal levels in the contralateral brains of both groups. Glutamate levels decreased rapidly after reperfusion in both the control and FK506 groups. The fact that basal levels of glutamate were constant throughout the experimental periods indicates equilibrium conditions in the contralateral brains and the validation of dual probe microdialysis techniques.

Some fundamental studies have shown that FK506 provides neuroprotective effects in animal models under conditions of cerebral ischemia [17,18]. There has been increasing interest in examining biochemical parameters to determine if FK506 can play a role in reducing brain damage. Preservation of extracellular pyruvate by naloxone in animal stroke models was observed in this laboratory [21]. FK506 may reserve glucose or preserve pyruvate and lactate within neuronal tissues for emergency energy needs during cerebral ischemia. However, much remains to be learned about the precise relationship between metabolic states and the critical thresholds at which the environmental status of the brain tissue becomes impaired and related to brain dysfunction.

Another major metabolic change during cerebral ischemia is acidosis. In general, there is a decline in high-energy phosphates, an increase in lactate, a decline in pH and a decline in the time courses of these changes in acute cerebral ischemia. These changes have been well documented in a number of animal models including rats and gerbils [4–7,14,15,21]. The lactate profile of the control group in this study was in agreement with that reported by this and other laboratories [4,9,21]. There was a significant difference in lactate profiles between the control and FK506 groups. It is not known whether the change in lactate is an epiphenomenon or is involved in neuronal death either directly or as a secondary aggravating factor. Increased lactate levels and decreased pyruvate production by tissues are demonstrated during cerebral ischemia, simply because of decreased regional cerebral blood flow and blood supply. Higher glucose levels were observed right after reperfusion in the FK506 group when compared to the control group. A proposed higher utilization rate of glucose also produced relatively higher concentrations of lactate in the FK506 group. The preservation of pyruvate was noted in the early reperfusion period. Increased brain glucose concentrations might indicate a reservoir of lactate and pyruvate sources during cerebral ischemia with FK506 pretreatment.

4. Conclusion

The microdialysis–LC technique demonstrates the time course of neurochemicals in the same animal because the animal does not have to be sacrificed to obtain neurochemical levels at each time point. This study intended to monitor chemical substances from both cortices in gerbils subjected to a focal cerebral ischemia/reperfusion. Changes in neurochemicals on the ipsilateral (ischemic) side can be compared to the baseline level and to the simultaneous recording on the contralateral (nonischemic) side in a dual microdialysis study. Microdialysis probes were performed appropriately with frequent in vitro microdialysis recovery tests. The dual probe microdialysis assay could be used to evaluate beneficial

therapeutic efficacy and/or adverse effects of an anti-ischemic drug, FK506. The present study demonstrated that the preservation of glucose and pyruvate and the enhancement of lactate by FK506 may be of neurological benefits. A complete explanation of FK506's beneficial effects is not yet possible, but anti-immune actions and their effects on energy related metabolites seem to be involved. The present study yielded useful information on the range and dynamic chemical changes following acute focal cerebral ischemia.

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References

[1] T. Sharp, T. Zetterstrom, H.G. Series, A. Carlsson, D.G. Grahame Smith, U. Ungerstedt, Life. Sci. 41 (1973) 869.
[2] U. Ungerstedt, C. Pycock, Bull. Schweiz. Akad. Med. Wiss. 30 (1974) 44.
[3] B.H. Westerink, P. Enrico, J. Feimann, J.B. De Vries, J. Pharmacol. Exp. Ther. 285 (1998) 143.
[4] U. Ito, M. Spatz, J.T. Walker Jr., I. Klatzo, Acta Neuropathol. Berl. 32 (1975) 209.
[5] T. Yanigihara, Stroke 9 (1978) 155.
[6] A. Crockard, F. Lannotti, A.T. Hunstock, R.D. Smith, R.J. Harris, L. Symon, Stroke 11 (1980) 494.
[7] K. Kahn, Neurology 22 (1972) 510.
[8] S. Levine, H. Payan, Exp. Neurol. 16 (1966) 255.
[9] D.E. Levy, J.B. Brierley, Exp. Arch. Neurol. 28 (1973) 503.
[10] F.C. Cheng, J. Wang, D.Y. Yang, Neurochem. Res. 25 (2000) 1089.
[11] L. Persson, L. Hillered, J. Neurosurg. 76 (1992) 72.
[12] T. Back, M. Hoehn, G. Mies, E. Bush, B. Schmitz, K. Kohno, K.A. Hossmann, Ann. Neurol. 47 (2000) 485.
[13] M.I. Sweeney, Neurosci. Rev. 21 (1997) 207.
[14] L. Hillered, A. Hallstrom, S. Segersvard, L. Persson, U. Ungerstedt, J. Cereb. Blood Flow Metab. 9 (1989) 607.
[15] B.A. Mizock, J.L. Falk, Crit. Care Med. 20 (1992) 80.
[16] J. Sharkey, S.P. Butcher, Nature 371 (1994) 337.
[17] S.P. Butcher, D.C. Henshall, Y. Teramura, K. Iwasaki, J. Sharkey, J. Neurosci. 17 (1997) 6939.
[18] J. Liu, J.D. Farmker, W.S. Lane, J. Friedman, I. Weissman, S.L. Schreiber, Cell 66 (1991) 807.
[19] F.C. Cheng, L.L. Yang, D.Y. Yang, Y.H. Tsai, C.W. Lee, S.H. Chen, J. Chromatogr. A. 870 (2000) 389.
[20] L. Persson, L. Hillered, J. Neurosurg. 76 (1992) 72.
[21] C.J. Chen, F.C. Chen, S.L. Liao, W.Y. Chen, N.N. Lin, J.S. Kuo, Neurosci. Lett. 287 (2000) 113.