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Metabolic and transmitter changes in core and penumbra after middle cerebral artery occlusion in mice

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Abstract

Middle cerebral artery occlusion (MCAO) is a popular model in experimental stroke research and causes prominent ischemic damage in the forebrain. To characterize metabolic changes induced by MCAO, we have induced permanent MCAO in mice that were implanted with a microdialysis probe in either striatum or hippocampus. Immediately after the onset of ischemia, glucose levels dropped to <10% of basal values in the striatum while they dropped to 50%, and recovered thereafter, in hippocampus. Extracellular levels of glutamate rose 80-fold in the striatum but only 10-fold, and in a transient fashion, in hippocampus. In striatum, release of acetylcholine briefly increased, then dropped to very low values. Both glycerol and choline levels increased strongly during ischemia in the striatum reflecting membrane breakdown. In hippocampus, glycerol increased transiently while the increase of choline levels was moderate. Taken together, these observations delineate metabolic changes in ischemic mouse brain with the striatum representing the core area of ischemia. In comparison, the dorsal hippocampus was identified as a brain area suitable for monitoring metabolic responses in the penumbra region.

Keywords

Choline; glucose; glutamate; glycerol; hippocampus; microdialysis; striatum

1. Introduction

Stroke is a major reason for death and disability in humans and, due to limited treatment options, is one of the focus areas of present biomedical research. Occlusion of the middle cerebral artery (MCAO) is a common cause of stroke in humans. In experimental research, MCAO is considered one of the best models to mimic human ischemic stroke and has been used in numerous studies in gerbils and rats (Carmichael, 2005). More recently, MCAO in mice has become a popular experimental model, mainly due to the ease of generating genetically

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modified animals in a murine background. The thread model of MCAO is popular because transient as well as permanent MCAO can be induced (Arumugam and Mattson, 2006).

The acute pathophysiological changes that occur in the ischemic brain are relatively well known and include loss of energy substrates (glucose and oxygen), loss of ATP, depolarization of brain cells and increase of extracellular potassium levels (Lo et al., 2003; Mehta et al., 2007). An important mediator of neuronal degeneration is the excitatory transmitter glutamate which is released in large quantities during ischemia from neurons and astrocytes and which causes cellular overload of calcium mainly through its action on calcium-permeable NMDA receptors (Lipton, 1999). Calcium overload during excitotoxicity leads to necrosis and breakdown of cellular structures including proteins, DNA and membrane phospholipids (Lipton, 1999). Besides glutamate, acetylcholine is another major excitatory transmitter in the brain and contributes to neuronal cell death, e.g. when acetylcholinesterase is inhibited (Solberg and Belkin, 1997).

An important concept in stroke research is the distinction between the “core” of the infarct and the “penumbra”, the area that surrounds the core. The almost complete loss of perfusion in the core area causes an almost complete loss of energy supply for neurons and neuronal death, a situation that may be delayed by neuroprotective drugs but hardly prevented. In contrast, the penumbra signifies brain tissue which remains partially perfused – usually through collateral vessels – and which degenerates in a more delayed fashion involving prominent apoptotic cell death (Mattson et al., 2001; Hara and Snyder, 2007). The visualization of the penumbra has been made possible using modern MRI and PET techniques (Guadagno et al., 2004). Importantly, most researchers believe that cell death in the penumbra can be prevented with suitable neuroprotective drugs.

Microdialysis is an experimental tool that allows access to the extracellular space of the brain (Bourne, 2003). As several pathophysiological markers of ischemic brain can be monitored in extracellular fluid, we used microdialysis in the present study to follow changes of glucose, the primary fuel for energy metabolism, and of the excitatory neurotransmitters glutamate and acetylcholine. In addition, we monitored cellular damage through the levels of glycerol and choline, two products of membrane breakdown. The experiments were performed in mouse striatum – a core area for ischemic damage – and mouse hippocampus, an area which we show - based on our neurochemical measurements - to belong to the penumbra. Our results demonstrate distinct differences between core and penumbra to ischemic damage and should thus facilitate future studies on the efficacy and mechanism of action of potential neuroprotective drugs.

2. Results

2.1 Probe placement

Fig. 1 illustrates TTC staining of striatal and hippocampal areas of the brain 24 hours after permanent MCAO. The placement of the microdialysis probes is indicated by black lines. It is evident that the striatal probes were located in the core region of ischemia while TTC staining in the hippocampus indicates moderate damage to this area and suggests that the hippocampal probe may sample from penumbral area. This hypothesis was tested by monitoring metabolic markers as described below.

2.2 Energy metabolites: glucose and lactate

Basal levels of metabolites and neurotransmitters in mouse striatum are given in Table 1, together with an estimate of the extracellular concentrations as calculated from the in vitro-recoveries. Basal levels of glucose before ischemia were $158 \pm 5 \mu\text{M}$ (N=41), and this value

remained unchanged in sham-operated mice (Fig. 2). MCAO caused an immediate decline of striatal glucose levels to values between 5–10% of baseline values within 20 minutes (Fig. 2). In the hippocampus, glucose levels dropped to 40% of control but recovered slowly to about 75% of controls indicating sustained partial perfusion in this area (Fig. 2).

Basal levels of lactate were $97 \pm 7 \mu\text{M}$ ($N=30$). Lactate levels were significantly increased during ischemia, however we noted that lactate rose several-fold already upon exposure of the mice to the anesthetic gases (data not shown). Since we could not distinguish increases of lactate due to anesthesia from those due to ischemia, we do not present lactate data in this manuscript. Current work in our laboratory is aimed at understanding the effect of anesthetic gases on lactate release.

2.3 Extracellular levels of glutamate

The extracellular concentration of glutamate in the striatum was $350 \pm 40 \text{ nM}$ ($N=41$; Table 1) and remained at that level in sham-operated mice (Fig. 3). MCAO caused an immediate, very prominent increase of striatal glutamate by (on average) 8,000% (Fig. 3). Glutamate levels decreased slowly thereafter but remained elevated by more than 30fold after 3 hours. In the hippocampus, glutamate was also strongly increased after MCAO by about 10-fold but recovered after 60 minutes to values that were only 2–4fold above baseline levels (Fig. 3).

2.4 Extracellular levels of acetylcholine

Acetylcholine (ACh) was only measured in the striatum because hippocampal levels were too low for detection. Striatal levels of ACh were $0.55 \pm 0.05 \mu\text{M}$ ($N=39$; Table 1). In the first 30 min after MCAO, acetylcholine levels rose to about 150% of controls before they dropped to 10–15% of controls after 50–60 minutes (Fig. 4). No recovery of ACh levels was seen during the period of measurement (3 hours).

2.5 Choline and glycerol

Choline and glycerol are both indicators of membrane breakdown (Klein, 2000). Basal levels of choline before ischemia were $0.49 \pm 0.05 \mu\text{M}$ ($N=40$; Table 1), and this value decreased slightly during the period of measurement. MCAO caused an immediate, 5 to 10-fold increase of striatal choline levels over a period of 2–3 hours (Fig. 5). Hippocampal choline levels, in contrast, rose only slightly to 200% of baseline over a period of 60–120 min (Fig. 5).

Basal levels of glycerol before ischemia were $4.0 \pm 0.4 \mu\text{M}$ ($N=37$; Table 1), and this value remained largely unchanged in sham-operated mice (a minor increase was seen after 30–40 min which was not significant). In the striatum, glycerol levels increased 6-fold after MCAO and remained high for 3 hours (Fig. 6). In the hippocampus, glycerol levels were increased 4-fold after 30–40 min but then declined although they remained elevated above baseline for the time of measurement (Fig. 6).

3. Discussion

3.1 Microdialysis and the MCAO stroke model

Microdialysis is a unique technique to gain access to the extracellular space of the brain, and the combination of microdialysis and stroke induction by MCAO allows real-time monitoring of metabolic changes in the brain in situ. This combined method has previously been used to investigate metabolic responses to ischemia in vivo, with most studies carried out in rats and gerbils but some studies also addressing primates and humans (Enblad et al., 2001; Berger et al., 2004). Most early studies focused on glutamate and amino acids (Benveniste et al., 1984; Hillered et al., 1989) whereas later studies also measured glucose, lactate/pyruvate ratio, or purines such as adenosine (Melani et al., 1999; Lin et al., 2002). There was only one study, to

our knowledge, that looked at cholinergic parameters in stroke but that study was performed using an ischemia/reperfusion model (Bertrand et al., 1993). Relatively few studies were done in the mouse, and all mouse studies so far focused on amino acids (Shimizu-Sasamata et al., 1998; Mitani and Tanaka, 2003). The reason for the scarcity of murine studies may be due to the technical challenge of combined surgery and microdialysis in very small animals.

We have recently established a procedure in which the microdialysis probe was implanted one day before MCAO was induced, and the present manuscript reports the first results from this approach. As we applied anesthetic gases (nitrous oxide/isoflurane) for the surgeries, we were able to follow metabolic markers post ischemia in mice that were awake for most of the post-ischemia interval of three hours. As a drawback of our procedure, we noted that lactate levels were strongly increased by anesthesia, and we could not purposefully use lactate measurements for monitoring ischemic events. On the other hand, several popular injection anesthetics are known to influence parameters such as glucose (ketamine/xylazine) and acetylcholine (barbiturates) so that compromises must be made for the interpretation of findings in anesthetized animals.

3.2 Effects of MCAO in the striatum

Our study compares metabolic changes in two brain regions of the mouse, striatum and hippocampus. The striatum is almost exclusively supplied by the middle cerebral artery, and accordingly, MCAO caused severe metabolic changes in this region (“core”). Immediately after stroke, glucose levels dropped rapidly to less than 10–15% of baseline values. This is likely due to rapid consumption of local glucose in the tissue and impairment of blood flow which reduces supply of new glucose moieties (Fig. 2). In agreement with earlier reports (Shimizu-Sasamata et al., 1998; Mitani and Tanaka, 2003), glutamate increased dramatically (more than 100fold in some animals; Fig. 3) indicating cellular release and lack of re-uptake which is hampered by lack of ATP and breakdown of membrane potentials in both neurons and glial cells (Hertz, 2008). Cellular release of glutamate, as observed in this and several other studies, is mainly due to release of metabolic pools from neuronal and glial cell bodies while the transmitter pool of glutamate contributes only a small fraction of early release (Danbolt, 2001). ACh, in contrast, is only formed in cholinergic nerve endings and has no metabolic role. Our data show a biphasic release of the neurotransmitter ACh: an early phase of increased release which is probably due to ischemic depolarisation of cholinergic nerve endings and a later phase during which ACh levels drop dramatically (Fig. 4). During prolonged ischemia, previously released ACh will be hydrolyzed by acetylcholinesterase and resynthesis of ACh will not occur due to lack of glucose and ATP. Both glucose and ATP are required for the synthesis of acetyl-CoA, a precursor of ACh, which will be rate-limiting for ACh synthesis in ischemia. In contrast, choline, the other precursor of ACh, is in rich supply (see below). It is notable that brain ACh synthesis strongly declines in brain ischemia whereas it was unaffected by severe hypoglycemia induced by insulin administration (Umegaki et al., 2006).

Glycerol and choline are indicators of phospholipid hydrolysis and therefore allow real-time monitoring of membrane breakdown under pathological conditions. The increase of these metabolites is initiated by calcium-dependent hydrolysis of membrane phospholipids. Choline release, for instance, is secondary to breakdown of phosphatidylcholine (PC), the major phospholipid in the brain, by phospholipase A₂ followed by hydrolysis of glycerophosphocholine to phosphocholine and free choline (Klein, 2000; Phillis and O'Regan, 2004). Glycerol formation can be induced by ischemic breakdown of any glycerol-containing phospholipid, with PC and phosphatidylethanolamine being the major species (Frykholm et al., 2001). In the present experiments, cellular damage is reflected by the huge increases of free glycerol and free choline (Figs. 5 and 6). Neither choline nor glycerol levels recover in

the striatum, again supporting the idea that lack of energy makes reuptake of precursors and resynthesis of vital cellular molecules impossible.

3.3 Effects of MCAO in the hippocampus

In sharp contrast to the data obtained in striatum, hippocampal metabolites show signs of recovery. Maximum changes of hippocampal metabolite levels are observed at 20 min (glucose and glutamate) or 30–40 min for the delayed release of glycerol from phospholipids; however, in all of these cases, recovery occurs and the metabolite levels return towards baseline levels. The exception to this rule is choline which displays a slow but constant formation in ischemic tissue, with no indication of recovery (Fig. 5). It should be noted, however, that none of the hippocampal metabolites actually reaches control levels during the time of observation (3 hours past MCAO) indicating ongoing cellular stress. It will be interesting to see in future studies how neuroprotective agents modify tissue levels of these metabolites.

3.4 Conclusion

In the present study, we have used microdialysis to monitor changes of extracellular metabolites after stroke induced by MCAO. While the changes of glucose and glutamate in the striatum were predictable, we observed a previously unknown biphasic release of the neurotransmitter acetylcholine and dramatic increases of metabolites such as choline and glycerol which reflect membrane breakdown and cellular degeneration. The hippocampus, in contrast, while showing similar changes as the striatum in the first 20–30 min past ischemia, seemed to recover thereafter and evidently represents penumbral tissue. As the penumbral area is the major target for neuroprotective drugs, monitoring metabolic parameters by microdialysis in the hippocampus will be a useful tool to investigate effectiveness and mechanisms of action of potential neuroprotective drugs.

4. Experimental procedures

4.1 Animals

Female CD-1 mice (26–32g; Charles River, Wilmington, DE) were kept under standardized light/dark (12h), temperature (22°C) and humidity (70 %) conditions, with mouse chow and water available ad lib. All animal procedures were in accordance with NIH regulations and were registered with the Institutional Animal Care and Use Committee of TTUHSC.

4.2 Implantation of microdialysis probe

Mice were anesthetized with isoflurane (induction dose 3%, maintenance dose 1–1.5% v/v) in a 30%/70% mixture of oxygen and nitrous oxide and placed in a stereotaxic frame. Self-made, I-shaped, concentric dialysis probes with an exchange length of 1 mm (for striatum) or 2 mm (for hippocampus) were implanted either in the left striatum or hippocampus using the following coordinates (from bregma): striatum, AP +0.5 mm; L +2.2 mm; DV –3.8 mm; hippocampus, AP –2.0 mm; L +2.0 mm; DV –2.3 mm (Franklin and Paxinos, 1997). A piece of tubing was fixed at AP –1.0 mm; L +3.5 mm for the monitoring of blood flow during MCAO surgery. Mice were allowed to recover over night, and the MCAO experiment was carried out on the day after probe implantation.

4.3 Ischemic stroke by middle cerebral artery occlusion

In vivo-ischemia in the brain was induced as described in detail previously (Mdzinariashvili et al., 2005). Briefly, female CD-1 mice were anesthetized with 1–1.5 % isoflurane in 30% O₂/70% N₂O. Throughout surgery, temperature was maintained at 37°C by a thermostatic blanket (rectal thermometer), and cerebral blood flow was monitored by laser Doppler flowmetry (Moor Instruments). The skin was incised, and the left occipital and superior thyroid

artery, branches of the external carotid artery (ECA), as well as the pterygopalatine artery were exposed, electrocoagulated, and cut. After occlusion of the common carotid artery by microclip, the left ECA was ligated, coagulated and cut distally to the cranial thyroid artery. A 15 mm monofilament nylon suture (5-0, Harvard Apparatus, Holliston, MA, USA; diameter of the heat-rounded tip: 0.2–0.3 mm) was inserted into the ECA and gently advanced through the internal carotid artery until its tip occluded the origin of the MCA. Correct placement of the suture was indicated by a sudden drop of the local cortical blood flow in the left MCA territory to 15–20% of basal flow as monitored by laser-Doppler flowmetry. After successful occlusion, the monofilament was secured in place with ligature, and the skin incision was closed by surgical clips.

In selected mice (N=6), blood was withdrawn before MCAO and 1 h after MCAO and physiological parameters were measured. Before MCAO, blood pH was 7.41 ± 0.03 and hematocrit was $51 \pm 3\%$. Plasma sodium was 144 ± 3 mM, potassium 5.3 ± 0.4 mM and chloride 108 ± 4 mM. pO_2 was 126 ± 12 mm Hg and pCO_2 was 38 ± 5 mm Hg. All values were within reference range except for the elevated oxygen partial pressure which was due to the increased oxygen content in the gas mixture (30% vs. 21 % in air). One hour after MCAO, none of these values was significantly changed (data not shown).

Perfusion of the microdialysis probe was started two hours before MCAO, was sustained while the MCAO surgery was performed, and was continued until 3 hours after MCAO. The perfusion fluid was artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ with 1 μ M neostigmine added to inhibit acetylcholine hydrolysis). The perfusion rate was 2 μ l/min, and efflux from the microdialysis probe was collected in intervals of 10 minutes.

Animals were allowed to recover from anesthesia after MCAO, immediately after closure of the skin wound, and took 15–30 min to regain full consciousness. MCAO was sustained for a period of 24 h, after which the animals were deeply anesthetized with isoflurane and euthanised by decapitation. The brains were quickly removed, sectioned coronally into 1 mm slices, and stained with 2,3,5-triphenyl-tetrazolium chloride (TTC) (Mdzinarishvili et al., 2005). TTC staining was used to verify successful stroke (Fig. 1) and to test for correct probe locations.

4.4 Chemical analysis of microdialysates

ACh and Ch in dialysates were determined by microbore HPLC-ECD using a metal-free system from Bioanalytical Systems (West Lafayette, IN). The procedure was performed as previously described (Hartmann et al., 2007). At an injection volume of 5 μ l, the detection limit of this system was 10–20 fmol/injection.

Metabolite concentrations in the microdialysis samples were determined by a CMA 600 microanalyzer (CMA, Stockholm, Sweden) using a kinetic photometric assay as described by the manufacturer. The following metabolites were measured (lower limits of detection in parentheses): glucose (1 μ M), lactate (1 μ M), glutamate (0.02 μ M) and glycerol (0.4 μ M). *In vitro*-recoveries were determined by dialysis of analytes from unstirred vial containing a fixed concentration (100-times the detection limit) of analytes.

4.5 Statistical analysis

Data are given as mean $\pm$ S.E.M. of *N* experiments. Time courses of microdialysis data (Figs. 2–6) were compared by two-way ANOVA. Statistical significance was derived from the column factor comparing the two curves (software: GraphPad Prism 4.0).

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Abbreviations

ACh	acetylcholine
MCAO	middle cerebral artery occlusion

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Fig. 1. Location of microdialysis probes. - Brain slices were stained with TTC in the area of the striatum (upper picture) and hippocampus (lower picture). Location of the probes was as indicated. Dialysis membranes were protected with silicon for 1 mm to avoid dialysis from cortical areas. Remaining exchange lengths in the striatum and hippocampus were approx. 2 mm and 1 mm, respectively.

Fig. 2.

Changes of glucose levels after MCAO measured by microdialysis. - The figure shows extracellular concentrations of glucose measured from 60 min before induction of stroke (MCAO, at time zero) to 120 min past stroke. Experiments were done in striatum ("core", N=8) and hippocampus ("penumbra", N=8) and were compared to sham-operated mice that did not sustain a stroke ("sham", N=8). Data are percentages (means $\pm$ S.E.M.) of basal release which was determined as average efflux from the measured samples prior to treatment (Table 1). Statistical analysis (two-way ANOVA, GraphPad Prism®): Glucose (sham) vs. glucose (core), $F_{1,280}=978.0$; $p<0.0001$. Glucose (sham) vs. glucose (penumbra), $F_{1,280}=65.7$; $p<0.0001$. Glucose (core) vs. glucose (penumbra), $F_{1,280}=107.5$; $p<0.0001$.

Fig. 3.

Changes of glutamate levels after MCAO measured by microdialysis. - The figure shows extracellular concentrations of glutamate measured from 60 min before induction of stroke (MCAO, see arrow) to 120 min past stroke. Experiments were done in striatum ("core", N=8) and hippocampus ("penumbra", N=8) and were compared to sham-operated mice that did not sustain a stroke ("sham", N=8). Data are percentages (means $\pm$ S.E.M.) of basal release which was determined as average efflux from the measured samples prior to treatment (Table 1). Statistical analysis (two-way ANOVA, GraphPad Prism®): Glutamate (sham) vs. glutamate (core), $F_{1,280}=114.2$; $p<0.0001$. Glutamate (sham) vs. glutamate (penumbra), $F_{1,280}=30.12$; $p<0.0001$. Glutamate (core) vs. glutamate (penumbra), $F_{1,280}=104.4$; $p<0.0001$.

Fig. 4.

Release of acetylcholine measured by microdialysis. - The figure shows extracellular concentrations of acetylcholine (ACh) measured from 60 min before induction of stroke (MCAO, see arrow) to 120 min past stroke. Experiments were done in striatum ("core", N=8) and were compared to sham-operated mice that did not sustain a stroke ("sham", N=6). Data are percentages (means $\pm$ S.E.M.) of basal release which was determined as average efflux from the measured samples prior to treatment (Table 1). Statistical analysis (two-way ANOVA, GraphPad Prism®): ACh (core) vs. ACh (sham), $F_{1,297}=138.4$; $p<0.0001$.

Fig. 5.

Choline levels measured by microdialysis. - The figure shows extracellular concentrations of choline measured from 60 min before induction of stroke (MCAO, see arrow) to 120 min past stroke. Experiments were done in striatum ("core", N=8) and hippocampus ("penumbra", N=6) and were compared to sham-operated mice that did not sustain a stroke ("sham", N=6). Data are percentages (means $\pm$ S.E.M.) of basal release which was determined as average efflux from the measured samples prior to treatment (Table 1). Statistical analysis (two-way ANOVA, GraphPad Prism®): Choline (sham) vs. choline (core), $F_{1,336}=135.7$; $p<0.0001$. Choline (sham) vs. choline (penumbra), $F_{1,277}=107.4$; $p<0.0001$. Choline (core) vs. choline (penumbra), $F_{1,336}=91.8$; $p<0.0001$.

Fig. 6.

Changes of glycerol levels after MCAO measured by microdialysis. - The figure shows extracellular concentrations of glycerol measured from 60 min before induction of stroke (MCAO, see arrow) to 120 min past stroke. Experiments were done in striatum ("core", N=8) and hippocampus ("penumbra", N=8) and were compared to sham-operated mice that did not sustain a stroke ("sham", N=6). Data are percentages (means $\pm$ S.E.M.) of basal release which was determined as average efflux from the measured samples prior to treatment (Table 1). Statistical analysis (two-way ANOVA, GraphPad Prism®): Glycerol (sham) vs. glycerol (core), $F_{1,240}=216.5$; $p<0.0001$. Glycerol (sham) vs. glycerol (penumbra), $F_{1,240}=82.2$; $p<0.0001$. Glycerol (core) vs. glycerol (penumbra), $F_{1,280}=61.1$; $p<0.0001$.

Table 1

Concentration of metabolites in mouse striatum under basal conditions

Parameter	Concentration in dialysate	Recovery in vitro	Extracellular concentration ¹
Glucose	158 ± 5 µM	6.3 %	2.51 mM
Lactate	97 ± 7 µM	9.2 %	1.05 mM
Glutamate	0.35 ± 0.04 µM	22 %	1.61 µM
Acetylcholine ²	0.55 ± 0.05 µM	9.5 %	5.79 µM
Choline	0.49 ± 0.05 µM	11 %	4.45 µM
Glycerol	4.00 ± 0.44 µM	14 %	28.5 µM

¹ Extracellular concentrations were estimated as “concentrations in dialysate” divided by “recovery in vitro”.

² in the presence of 1 µM neostigmine