Astrocyte metabolism following focal cerebral ischemia

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Abstract

Stroke is one of the leading causes of disability and death. Most often, stroke results from blockage of an artery in the brain leading to tissue infarction within the perfusion territory of the affected vessel. Despite the severity of the insult, many cells are not irreversibly damaged within the first few hours and can be rescued by early restoration of blood flow or other interventions. Astrocyte, the most numerous cells in the brain, normally perform many functions that are essential for neuronal viability. Thus, stimulation of key astrocyte properties in ischemic or post-ischemic brain could potentially contribute to neuroprotection. However, at present, there is very little understanding of either the response of astrocytes to cerebral ischemia or the extent to which these cells can recover function if blood flow is restored.

The main aim of the project was to assess key metabolic properties in astrocytes during early reperfusion following unilateral occlusion of the middle cerebral artery (MCA) in rats. Astrocytic oxidative metabolism was assessed from the incorporation of radiolabel from [1-^{14}C]acetate into glutamine, an activity that is essentially specific for these cells. Striatal tissue from the hemisphere subjected to ischemia showed substantial decreases in ^{14}C-glutamine production at 1 hour of reperfusion following either 2 or 3 hours of ischemia. In contrast, this activity was almost fully preserved for at least 4 hours in parts of the cerebral cortex that had been subjected to more moderate ischemia, even when the duration of ischemia was sufficient to induce infarction in this region. The production of ^{14}C-glutamine was also not significantly affected in cortical tissue exposed to more severe ischemia but this measure was much more variable between animals. These findings demonstrate regional differences in the response of astrocytes to focal ischemia and provide evidence that most cortical astrocytes remain viable and metabolically active for many hours, even in tissue destined to become infarcted.

To further evaluate metabolic recovery in the post-ischemic brain, the production of ^{14}C-glutamate and ^{14}C-glutamine from [U-^{14}C]glucose was assessed. Neurons are responsible for most of the ^{14}C-glutamate generation whereas ^{14}C-glutamine is produced in astrocytes from glutamate of neuronal and astrocytic origin. Marked reductions in the labeling of both amino acids were observed in all regions of the MCA territory during early reperfusion after either 2 or 3 h ischemia irrespective of whether the tissue would become infarcted. These results provide evidence for widespread depression of glucose metabolism in neurons and altered metabolic interactions with astrocytes. Interestingly, this reduction in glucose metabolism was not associated with substantial changes in tissue phosphocreatine content and ATP:ADP ratio suggesting that energy requirements were reduced by the ischemia-reperfusion.

Increases in lactate content were detected during early reperfusion in tissue regions that would develop infarcts. This finding coupled with previous evidence for deleterious effects of lactic acid suggests that accumulation of this metabolite might promote cell death. An impairment of pyruvate oxidation or reduced clearance of lactate could contribute to the increased lactate. The mechanisms by which excess lactate is cleared from the brain are not known. We hypothesized that MCT4 is involved in the removal of lactate as this transporter isoform is responsible for lactate export from other tissues. Using immunogold cytochemistry, MCT4 was found to be densely expressed in the endfeet of glial cells facing blood capillaries and pial surface of the brain, suggesting an important role in the removal of excess lactate from the CNS. In future studies, the expression of MCT4 will be examined following ischemia to resolve whether an altered expression of this transporter may be one reason for the elevated lactate levels in the brain.

Key words: *Astrocyte, metabolism, focal cerebral ischemia, reperfusion, infarct, [1-^{14}C]acetate, [U-^{14}C]glucose, glutamine, glutamate, ATP, ADP, lactate, MCT4, immunogold cytochemistry.*

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Populärvetenskaplig sammanfattning på svenska

Stroke utgör som del i det kardiovaskulära sjukdomspanoramat en av de främsta orsakerna till funktionsnedsättning och död. Förutom det lidande som drabbar de enskilda patienterna och deras familjer, kostar följderna efter stroke samhället stora summor varje år i form av vård, rehabilitering och förlorade produktiva år. Stroke inträffar vanligtvis till följd av att en blodpropp fastnar i en av hjärnans artärer. Därmed minskar blodtillflödet (cerebral ischemi) till den del av hjärnan som försörjs av den aktuella artären. Det påverkade hjärnområdet området får då för lite syre ifrån blodet vilket i sin tur kan leda till akut energibrist i hjärnans olika celler. Detta leder i många fall till omfattande celldöd med funktionella konsekvenser som följd. I vissa fall kan man med proppupplösande behandling återfå blodflödet i det påverkade hjärnområdet men det förutsätter att behandlingen sätts in mycket snabbt efter insjuknandet. En annan möjlighet att minska skadans omfattning i den initiala fasen efter stroke kan i framtiden vara att ge ett farmakologiskt skydd, s.k. neuroprotektiv behandling, av hjärnans celler. För att sådan behandling skall vara framgångsrik krävs dock omfattande kunskap om hur hjärnans celler reagerar efter stroke. Skademekanismerna efter syrebrist är mycket komplexa och involverar förändringar i ett flertal av cellernas normala funktioner.

Astrocyterna är de vanligast förekommande cellerna i hjärnan och de har många viktiga roller som är intimt sammankopplade med nervcellernas funktioner och skydd. Genom att påverka vissa nyckelfunktioner hos astrocyterna har det visat sig vara möjligt att indirekt även påverka nervcellernas olika funktioner både i den normala situationen och efter olika skador och sjukdomar. Kunskapen kring dessa processer är dock fortfarande mycket begränsad. Det är av fundamental vikt att ämnesomsättningen i de enskilda hjärncellerna upprätthålls för att funktionell återhämtning skall kunna ske efter en stroke. Arbetet i den aktuella avhandlingen har i huvudsak fokuserats på hur astrocyternas ämnesomsättning förändras efter experimentell stroke genom temporär blockad av den mediala hjärnartären i råtta.

Genom att utnyttja en unik egenskap i astrocyternas metabolism har vi som första grupp specifikt visa hur dessa celler reagerar efter experimentell stroke. Våra studier visar att astrocyter i olika hjärnregioner har olika möjlighet att motstå effekterna av syrebrist. Astrocyterna i hjärnbarken uppvisade under flera timmar efter genomgången stroke en normal ämnesomsättning medan astrocyterna från striatum uppvisade tidiga tecken på sviktande funktion. Detta antyder att astrocyterna har olika motståndskraft mot syrebrist i olika hjärnregioner vilket i sin tur indikerar att de har olika specialiseringsgrad beroende på var i hjärnan man befinner sig.

I den efterföljande studien utvärderades glukosomsättningen i både neuron och astrocyter genom att studera produktionen av cellspecifika aminosyror. Till skillnad mot den tidigare studien noterade vi att det förelåg en signifikant reduktion av glukosomsättningen i alla de områden av hjärnan som hade involverats i av den cerebrala ischemin. Förändringen var särskilt stor hos neuronen. Genom att mäta olika energirika föreningar fann vi också tecken på ett lägre energibehov generellt i hjärnvävnaden vilket tolkades som ett tecken på anpassning till den rådande situationen.

I efterförloppet till den cerebrala ischemin fann vi också förhöjda laktathalter i de hjärnområden som senare skulle utveckla infarkt. Dessa resultat tillsammans med tidigare fynd angående olika cellskadande effekter av laktat skulle kunna tyda på en koppling till utvecklingen av cellskada efter ischemi. Laktat kan ackumuleras i hjärnvävnaden genom en försämrad oxidering av metaboliten pyruvat, alternativt en minskad transport ut ur hjärnvävnaden till cirkulationen.

Mekanismerna för laktat transport ut ur hjärnan är ofullständiga. Vi beslöt oss därför att kartlägga vilka mekanismer som vanligtvis ansvarar för utsöndringen av laktat från hjärnan. Genom avancerade elektronmikroskopiska studier visade det sig att astrocyterna uttrycker en specifik laktattransportör, MCT4, i änden på sina långa utskott som avslutas i kontakter vid blod-hjärn-barriären. Resultaten antyder att dessa transportörer kan vara viktiga för utsöndring av överflödigt laktat från hjärnvävnaden till blodbanan.

Genom att utnyttja vissa av astrocyternas unika egenskaper har vi för första gången i den intakta hjärnan kunnat visa att undergrupper av dessa celler har förmåga att motstå konsekvenserna av syrebrist under många timmar. Detta kan öppna för specifik farmakologisk intervention i syfte att direkt skydda överlevande astrocyter och därmed indirekt uppnå en gynnsam effekt på omgivande nervvävnad.

PAPERS INCLUDED IN THE THESIS

This thesis is based on the following papers:

Paper I Astrocyte function assessed from [1-^{14}C]acetate metabolism following temporary focal cerebral ischemia in the rat.
Thorén A.E., Helps S.C., Nilsson M., Sims N.R.
Journal of Cerebral Blood Flow and Metabolism 2005 Apr; 25 (4):440-50.

Paper II The metabolism of ^{14}C-glucose by neurons and astrocytes in brain subregions following focal cerebral ischemia in rats.
Thorén A.E., Helps S.C., Nilsson M., Sims N.R.
Journal of Neurochemistry 2006 May; 97 (4):968-78.

Paper III Specialized membrane domains for lactate transport at the blood-brain and blood-retinal interfaces: enrichment of MCT4 in glial endfeet membranes.
Thorén A.E., Sørbø J-G., Holen T., Moe S-E., Bergersen L.H., Ottersen O-P., Nilsson M., Nagelhus E.A.
Manuscript (2006).

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenine triphosphate
AQP4	Aquaporin 4
BBB	Blood Brain Barrier
$[Ca^{2+}]_i$	Intracellular calcium
CNS	Central Nervous System
EC	Electrochemical Detection
GFAP	Glial Fibrillary Acidic Protein
GS	Glutamine Synthetase
EC	Electrochemical Detection
HPLC	High Pressure Liquid Chromatography
MCA	Middle Cerebral Artery
MCT	Monocarboxylate transporter
NMR	Nuclear Magnetic Resonance
OGD	Oxygen Glucose Deprivation
OPA	o-phthaldialdehyde
PAG	Phosphate activated glutaminase
PC	Pyruvate carboxylase
PCr	Phosphocreatine
PDH	Pyruvate Dehydrogenase
ROS	Reactive Oxygen Species
TCA	Tricarboxylic acid
TTC	2,3,5-triphenyl 2-H-tetrazolium chloride

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INTRODUCTION

Stroke and related cerebrovascular disorders are a major cause of adult disability and death. Approximately 30 000 Swedish people are affected every year. The pattern and extent of neural cell loss is a key determinant of the long-term neurological consequences of these disorders. The development of cell death is influenced by the severity and duration of the ischemic insult as well as the brain areas affected (Lipton, 1999). Considerable progress has been made in identifying factors contributing to cell death following the interruption of blood flow. Nonetheless, the mechanisms of cell loss are far from completely defined. Advances in this area could lead to the development of novel therapeutic approaches to restrict cell loss and improve the prognosis for affected individuals.

Blockage of a vessel within the brain is the most common cause of stroke. Permanent occlusion usually leads to tissue infarction involving the death of essentially all cells in the area perfused by the affected vessel. The interruption of blood flow reduces the delivery of oxygen and glucose to the affected region, leading to cell dysfunction and death. The occlusion produces an area of severely ischemic tissue (focal region) and a region in which reductions in perfusion are less severe, known as the penumbra or perifocal tissue (Fig. 1, Siesjo, 1992; Lo et al., 2003). Damage develops initially in the focal area but spreads to also include the moderate ischemic region. Studies using animal models have shown that some treatments can reduce infarct formation in perifocal but not focal tissue indicating that different mechanisms contribute to cell loss in these regions (Dirnagl et al., 1990; Park and Hall, 1994). It is likely that the main reason for cell death in the focal region is lack of oxygen and glucose during permanent ischemia while the mechanisms leading to cell death in the perifocal tissue are more complex.

Reversal of the occlusion can occur spontaneously or as a result of thrombolytic treatment. Animal studies have shown that if the reversal occurs at an early time point (within one hour of occlusion), most cells in both the focal and perifocal regions survive (Kaplan et al., 1991; Memezawa et al., 1992; Anderson and Sims, 1999).

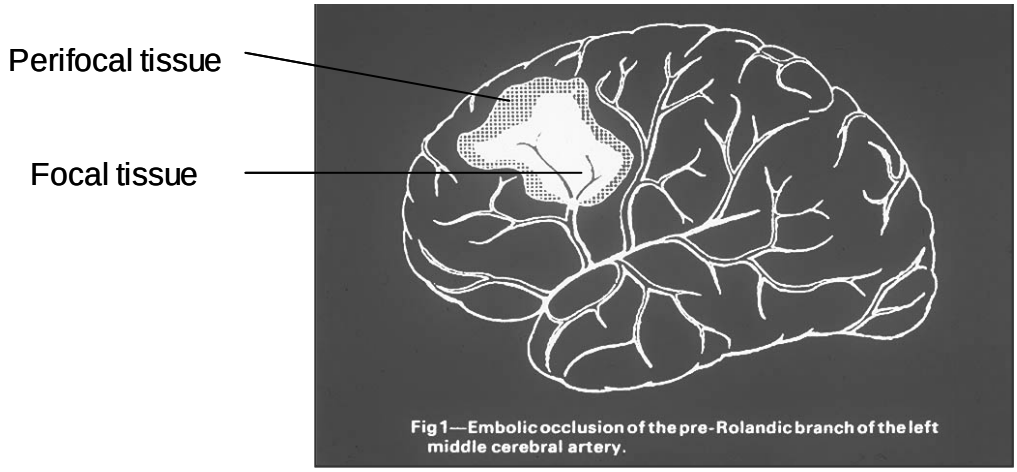

FIG. 1. The occlusion of the pre-Rolandic branch of the middle cerebral artery. The occlusion of the artery results in a severely ischemic core (focal tissue) and a moderate ischemic area (perifocal tissue, picture taken from Pulsinelli, 1992).

Extending the ischemic period to several hours induces infarct formation comparable to that with permanent ischemia. Probable factors contributing to cell death following reversal of the occlusion are likely to include oxidative stress, excitotoxicity and inflammatory responses (Siesjo 1992; Jean et al., 1998; White et al., 2000; Nishizawa, 2001; Sims et al., 2004; Starkov et al., 2004). Interestingly, studies in animal models indicate that much of the cell loss is not irreversibly determined at the onset of reperfusion, as a range of treatments within the first few hours of recirculation have been shown to greatly reduce infarct size (Markgraf et al., 1998; Yoshimoto and Siesjo, 1999; Yrjanheikki et al., 1999; Ginsberg et al., 2003; Xu et al., 2006).

At present, there is not a good understanding as to when or to what extent the different cells in the brain recover during early recirculation and which cell populations are affected first. This is critical information for trying to develop treatments that can be initiated upon restitution of blood flow to protect the cells after ischemia. Studies of the sequences of events during and after ischemia have focused on neurons. However, in recent years it has become increasingly clear that astrocytes, the most abundant population of cells in the brain, are necessary for brain function and that they are intimately linked to the neurons both structurally and functionally (Hansson et al., 2000; Walz, 2000; Nedergaard et al., 2003; Newman, 2003). Therefore, when astrocytes fail to survive or function, the survival of neurons is likely to be compromised. However, at present,

there is a limited understanding of the role of astrocytes in the post-ischemic brain and to what degree they ameliorate or exacerbate damage to neurons.

Astrocytes

Astrocytes are classically divided into three major types according to their morphology and spatial organization: protoplasmic astrocytes in grey matter, fibrous astrocytes in white matter and radial astrocytes surrounding ventricles (Privat et al., 1995). Astrocytes constitute the main population of glial cells in the brain and represent over 50% of the total cell number in the cerebral cortex and 20-30% of total cell volume (Bass et al., 1971; Tower and Young, 1973). These cells can be identified by staining for glial fibrillary acidic protein (GFAP), revealing star shaped morphologies. Recent studies using microinjections of single astrocytes has revealed "bush" shapes that are structurally arranged with minimal overlap and with specific territories, forming so called microdomains (Bushong et al., 2002, 2004; Wilhelmsson et al., 2004; Oberheim et al., 2006). These microdomains consist of fine, motile extensions that cover thousands of synapses, allowing dynamic interactions between the two cell types (Hirrlinger et al., 2004; Benediktsson et al., 2005). The population of astrocytes appears to be heterogenous. Astrocyte-specific genes possess strikingly varied regional patterns of expression, as demonstrated by mRNA microarrays (Bachoo et al., 2004). The heterogeneity of astrocytes is also supported by findings from mice expressing enhanced green fluorescent protein under the GFAP promoter (Nolte et al., 2001). This and other studies have revealed that approximately half of the cells show "typical" astrocyte properties, with extensive gap junction coupling, high expression of GFAP, low input resistance and very negative membrane potential (Matthias et al., 2003; Grass et al., 2004; Wallraff et al., 2004). A large proportion of the remaining astrocytes have low GFAP, larger input resistance, voltage-dependent K^+ and Na^+ currents and lower gap junction connectivity. These findings suggest that we have only started to unravel the complexity and functions of these cells.

Astrocytes were for many years a neglected component of the brain as they were only thought to have a physical supportive function. Now these cells are believed to perform many important tasks essential for normal brain function. *In vitro* and *in vivo* investigations have demonstrated that astrocytes express several receptors, ion channels and second messenger systems earlier

believed to present exclusively in neurons (Nilsson et al., 1991; Condorelli et al., 1999; Verkhratsky and Steinhauser, 2000; Muyderman et al., 2001; Haydon and Carmignoto, 2006). The processes that ensheath synapses have been found to be essential participants in regulating the components of the synaptic cleft and play an important role in the integration of information (Newman, 2003; Perea and Araque, 2006). Glutamate released at the synapses can induce astrocytic exocytosis of glutamate, modulating the activity and strength of the synapse (Bezzi et al., 2004; Liu et al., 2004). Thus, the synapse is now considered to consist of three units, the presynaptic and postsynaptic neuronal elements plus the astrocyte, together forming what has been termed the tripartite synapse (Takumi et al., 1998; Haydon and Carmignoto, 2006).

Clearance of glutamate from the extracellular space at the synapse, necessary for normal neuronal signalling, is primarily accomplished by Na^+ dependent transporters localized on astrocytes (reviewed in Danbolt, 2001). A large proportion of the glutamate taken up by the astrocytes is converted to glutamine by an enzyme, glutamine synthethase (GS), exclusively localized in astrocytes (Martinez-Hernandez et al., 1977). Glutamine, which does not act as a neurotransmitter, can be released back into the extracellular space and taken up by neurons. The neurons can then convert glutamine back to glutamate for replenishment of the neurotransmitter pool (Broer and Brookes, 2001; Chaudhry et al., 2002).

Astrocytes not only provide neurons with precursors for neurotransmitters, but are also assumed to deliver metabolic substrates so that the neurons can cope better with changing energy demands (Wiesinger et al., 1997; Dienel and Cruz, 2004). Pellerin and Magistretti suggested that the uptake of glutamate by astrocytes increase the intracellular Na^+ that activates the Na^+/K^+ ATPase, which in turn reduces the levels of ATP that stimulates glycolytic activity, initiating production and release of lactate from astrocytes (Fig. 2) (Pellerin and Magistretti, 1994). This enhanced production of lactate is proposed to support neuronal metabolism during neurotransmission. However, this model is controversial since it has not been demonstrated that neurons mainly metabolise lactate during activation and that lactate release is directly coupled to astrocyte intracellular Na^+ elevations. For example, *in vitro* studies have demonstrated that elevated extracellular K^+ increases astrocyte glycolysis and enhances lactate release (Walz and Mukerji, 1988). The metabolic responses of cultured astrocytes to glutamate are diverse. Several

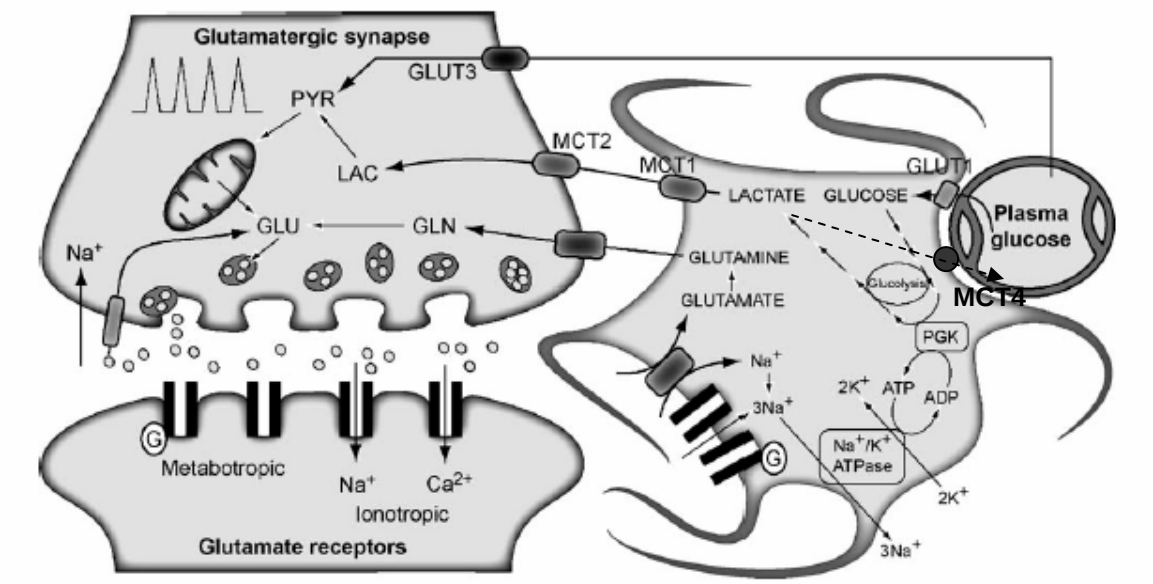

FIG. 2. A model for metabolic coupling between astrocytes and neurons, proposed by Pellerin and Magistretti (1994). Glutamate (GLU) released during neurotransmission is taken up by Na^+ co-transporters located on astrocytes. The intracellular Na^+ levels rise in astrocytes, activating Na^+/K^+ ATPase that consumes one ATP. The glutamate is converted to glutamine (GLN) by GS, consuming a second ATP molecule. The consumption of ATP stimulates glycolysis, producing 2 lactate (LAC) molecules that are extruded into the extracellular fluid and taken up by neurons. Abbreviations: GLUT1 and GLUT3, glucose transporters 1 and 3; MCT1 and MCT2, monocarboxylate transporter 1, 2 and 4; PGK, phosphoglycerate kinase; PYR, pyruvate. The figure is used with permission from Pierre Magistretti (1994) and Garcia-Martin (Cerdan et al., 2006).

laboratories have observed either no effect or a decrease in glucose utilization or lactate formation (reviewed in Deniel and Cruz, 2004). Evidence that might suggest a higher glycolytic capacity in astrocytes compared to neurons is their relatively higher release of lactate *in vitro* (Walz and Mukerji, 1988). Astrocytes are the main storage sites of glycogen and there is a substantial decrease in the content of this metabolite during neuronal activation (Swanson et al., 1992; Brown et al., 2003, 2004). Also, the extracellular lactate levels are increased during brain activation (Prichard et al., 1991; Hu and Wilson, 1997). Taken together, the available literature data suggest that there is a net production of lactate by astrocytes, although it remains to be determined to what extent neurons rely on lactate as a metabolic substrate.

Glial cells have also been suggested to deliver other substrates for neuronal metabolism. The enzyme pyruvate carboxylase (PC) is selectively expressed by glial cells. This enzyme catalyzes oxaloacetate production, enabling de novo synthesis of tricarboxylic acid (TCA) constituents (Yu et al., 1983; Shank et al., 1985). The selective localisation of PC suggests that astrocytes might

provide metabolites, such as citrate, α-ketoglutarate and malate, to replenish losses of TCA cycle intermediates in neurons (Shank and Campbell, 1984; Sonnewald et al., 1991).

Unlike most neurons, most astrocytes are highly coupled to each other through gap-junctions, forming syncytia in the CNS where substances ≤1 kDa can pass down their concentration gradient (Fischer and Kettenmann, 1985; Dermietzel et al., 1991; Zahs, 1998; Rouach et al., 2002). Through these gap junctions, specific messages can be delivered to neighbouring cells by $[Ca^{2+}]_i$ transients at varying frequencies. The transients are transmitted internally between the cells by the release of inositol 1,4,5-triphosphate ($Ins1,4,5P_3$) but also externally by the release of ATP from the astrocytes (Hagberg et al., 1998; Anderson et al., 2004). The $[Ca^{2+}]_i$ elevations can be elicited spontaneously, mechanically, and *in vivo* by glutamate (Venance et al., 1997; Wang et al., 2006 a, b; Zur and Deitmer, 2006). The frequency and intensity of these oscillations are encoded to give specific responses of the cells, where one example of such an event is the exocytosis of glutamate (Muyderman et al., 2001; Bezzi et al, 2004). Not only are these syncytia important for communication but are also essential components for maintaining the homeostasis of the extracellular fluid through buffering of substances such as K^+ and H^+ (spatial buffering) and the trafficking of glucose and other substances from the blood- brain interface (Newman, 1986; Clausen, 1992; Giaume et al., 1997; Morgello et al., 1995; Walz, 2000; Wallraff et al., 2006).

The astrocytes have processes that are directed towards microvessels. These form specialized structures that abut onto the perivascular membrane, so called endfeet (Fig. 3). These domains have been shown to be highly specialized, functionally as well as anatomically, and express specific proteins for uptake and release of substances into the blood. Typical of the endfoot membrane is the presence of high density orthogonal arrays of particles now known to contain the water channel aquaporin 4 (AQP4, Neely et al., 2001; Amiry-Moghaddam and Ottersen, 2003a).

The endfoot membrane also expresses the Kir4.1 K^+ channel, suggesting that they are essential components in the volume regulation and homeostasis of the brain (Price et al., 2002; Nagelhus et al., 2004). Recent evidence also suggests that the endfeet are important domains for the

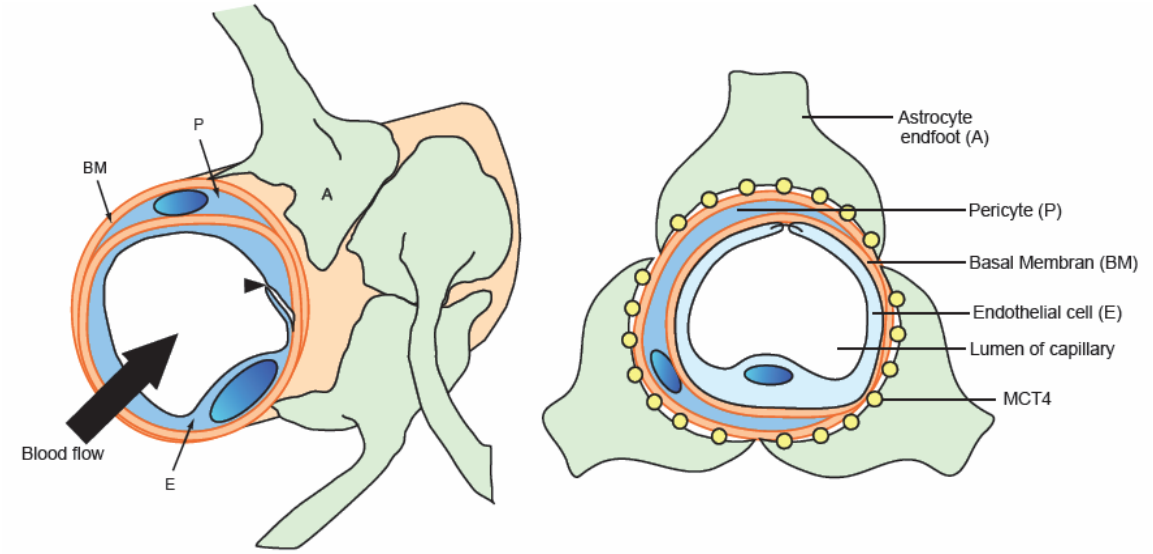

FIG. 3. The neurovascular unit.
Astrocytes have structures, so called endfeet, that abut onto blood vessels. These are highly specialized domains and express specific proteins, such as AQP4, Kir4.1 as well as MCT4 (paper III). The endothelial cells, pericytes and astrocyte endfeet form the neurovascular unit which is highly regulated to take up and release specific substances into the blood stream and regulate local cerebral blood flow. Arrowhead points at a tight junction between endothelial cells. Left figure is adapted with permission from Abbott et al. (1989).

formation and regulation of the blood brain barrier (BBB). The BBB is classically considered to be a physical and metabolic barrier restricting entry of blood-borne substances into the brain. The endothelial cells lining the brain capillaries in the brain have been widely accepted to constitute the most important component of the BBB due to their tight junctions between adjacent cells (Bradbury, 1985). There is now strong evidence, particularly from in vitro studies, that astrocytes can up-regulate many features of the endothelial cells, leading to improved tight junctions, increased expression of GLUT1 (glucose transporter) and P-glycoprotein (Hayashi et al., 1997; Schinkel, 1999; McAllister et al., 2001). Further, studies have shown that the perivascular membranes may be rate limiting when it comes to passage of water across the BBB suggesting that astrocyte membranes are critical and yet largely neglected components of the brain-blood interface (Amiry-Moghaddam et al., 2004). In addition, astrocyte endfeet participate in the regulation of blood flow and contribute importantly to the formation of BBB (Fig. 3) (Ramsauer et al., 2002; Takano, 2006). More investigations are needed to identify the roles of astrocytes at the blood-brain interface and the interactions between endfeet, endothelial cells and pericytes (Tilton et al., 1979; Schonfelder et al., 1998; Allt and Lawrenson, 2001).

It is well documented that astrocytes *in vivo* become "activated" in response to CNS injuries (astrocytosis). Initially, the cells undergo hypertrophy and changes in gene expression and up-regulation of intermediary filaments. This initial reactive response has been shown to have an important role for BBB repair, reduced brain edema and regulation of blood flow as demonstrated in transgenic mice (Bush et al., 1999; Faulkner et al., 2004). As the damage progresses toward infarction, a proportion of the surviving astrocytes in the periphery gradually transform into a scar that seals off the affected tissue (reviewed in Pekny and Nilsson, 2005). The scar may restrict further spreading of cell death but it appears that it in the later period restricts neuronal growth (McKeon et al., 1991; Pekovic et al., 2005). The regeneration of neurons following a CNS insult is improved in transgenic mice that lack GFAP and hence show reduced glial scar formation (Menet et al., 2001; Wilhelmsson et al., 2004). These studies have provided evidence that astrocytes play an important and active role in CNS injuries.

Astrocytes and ischemia

The initial response of the astrocytes following cerebral ischemia is likely to be important for neuronal protection. Studies have demonstrated that astrocytes contain greater concentrations of the antioxidant glutathione and enzymes involved in glutathione metabolism than neurons, making it likely that astrocytes protect neurons against oxidative stress generated following ischemia (Hjelle et al., 1994; Makar et al., 1994; Wilson, 1997; Fiskum et al., 2004). The excitatory amino acid glutamate is released in large quantities during ischemia, and the removal of this neurotransmitter, predominantly accomplished by astrocytes, is important for neuronal survival in the post-ischemic tissue (Stanimirovic et al., 1997; Romera et al., 2004). In contrast to these findings, astrocytes have been suggested to play a detrimental role following ischemia as the gap junctions may remain open (Martinez and Saez, 2000), allowing substances such as proapoptotic factors to spread through the syncytium thereby expanding the size of the infarct (Lin et al., 1998a).

At present, there is limited information on the function of astrocytes in the post-ischemic brain. Many studies identifying changes *in vivo* do not allow the consequences for different cell populations to be identified. For example, the production of nitric oxide and other free radicals can modify oxidative metabolism and impair ATP production (reviewed in Lo et al., 2003).

Similarly, changes have been found in mitochondrial properties that could further limit oxidative metabolism (Bogaert et al., 2000; Sims and Anderson, 2002). It is currently unknown to what extent astrocytes are affected by these changes and to what extent they recover functionally.

In vitro studies have provided substantial insight into the mechanisms governing the survival of astrocytes following simulated ischemia. These investigations have shown that astrocytes are generally more resistant than neurons to oxygen-glucose deprivation (OGD) (Shay and Ames, 1976; Goldberg and Choi, 1993; Sochocka et al., 1994). Most neurons in a cortical astrocytic-neuronal co-culture show signs of cell death after 60-70 min of OGD while astrocyte cultures require several hours to develop such extensive damage (Almeida et al., 2002). However, it appears that not all groups of astrocytes are similarly resistant to ischemic insults. Astrocytes cultured from different regions from the brain, such as cortex, striatum and hippocampus, seem to differ in the sensitivity to OGD (Zhao and Flavin, 2000; Xu et al., 2001). The *in vitro* studies have also provided a better understanding of what mechanisms influence astrocytic cell death. For example, a combination of hypoxia and acidosis has been found to be very effective in killing astrocytes (Giffard et al., 1990; Swanson et al., 1997; Bondarenko and Chesler, 2001).

Much of the information about the recovery of astrocytes *in vivo* has been provided by studies using immunohistological markers for astrocyte specific proteins. Most investigations suggest that astrocytes are better preserved than neurons in animal models of stroke (Chen et al., 1993; Li et al., 1995; Lee et al., 2003). These studies demonstrated preserved GFAP expression within the first 3 h of reperfusion after 2 h MCA occlusion and an increase in GS at 3 h (following 3 h occlusion) (Li et al., 1995; Lee et al., 2003). At later reperfusion periods, GFAP was increased in the peri-infarct areas, that later develop into the glial scar (Li et al., 1995). Neuronal markers were already reduced at 1 h in the striatum and progressively decreased at later time periods. In contrast to these studies, Liu et al. (1999) reported that deterioration of some astrocyte markers preceded that of neuronal markers. However, several of these observations were based on mRNA levels rather than protein expression which might explain the discrepancy in conclusions. Another investigation indicates that not all astrocyte sub-populations are equally resistant to ischemia. Lukaszevicz et al. (2002) reported that protoplasmic astrocytes lost their integrity faster than that of fibrous astrocytes, which partially could explain regional susceptibility to ischemia.

The above studies have provided the first evidence about astrocytes in the post-ischemic brain. However, they offer little insight into the preservation of astrocyte function and the metabolic changes that occur in these cells following ischemia.

To obtain a direct measure of the extent to which astrocytes survive during focal ischemia and are able to restore key properties in the post-ischemic brain, we evaluated the oxidative metabolism of ^{14}C-acetate based on accumulation of radiolabel into glutamine in an in vivo model of stroke (paper I). ^{14}C-acetate is selectively taken up by these cells, converted to acetyl CoA and further metabolized via the tricarboxylic cycle (TCA). Glutamine is rapidly labeled by the action of glutamine syntethase (GS) that is specifically expressed by astrocytes. To provide further evidence about the recovery of the post-ischemic tissue, the metabolism of ^{14}C-glucose was assessed by the incorporation of radiolabel into glutamate and glutamine (paper II). ^{14}C-glutamate is almost exclusively generated by neurons while ^{14}C- glutamine is produced by astrocytes using glutamate of both neuronal and astrocytic origin. Thus, the production of ^{14}C-glutamine measure can be potentially influenced by changes in both cell populations. ATP, ADP, phosphocreatine (PCr) and lactate content of the tissue was also investigated as a further measure of cell recovery and to help with the interpretation of the metabolic studies. Several regions from the post-ischemic tissue were investigated and the results related to the severity and duration of ischemia to which the area had been exposed. One of the findings from this study (paper II) showed that there were large accumulations of lactate that were related to the pattern of cell death that subsequently develops. In fact, lactate and acidosis have been identified to be factors that are correlated to the size of infarction. Hyperglycemia is associated with increased lactate and acidosis levels following temporary focal cerebral ischemia (reviewed in Kagansky et al., 2001) and animals show increased tissue infarction compared to the normoglycemic animals (de Court et al., 1989; Gisselsson et al., 1999). One of the mechanisms that are believed to aggravate the neuronal and glial cell death is elevated lactate levels and acidosis. The enhanced acidosis may exaggerate ischemic damage by increased free radical formation, perturb intracellular transduction pathways and activate endonucleases (Siesjo et al., 1996). Evidence that supports the negative effects of lactate and acidosis is the stroke-like infarctions that develop after intercerebral injections of sodium lactate solutions (pH 4.5-5.3) (Kraig et al., 1987; Petito et al.,

1987). Information is scarce as to how excess lactate is cleared from the brain. This lack of information prompted us to undertake an analysis of lactate transporters in brain tissue (paper III).

Lactate transporters

The transport of monocarboxylic acids such as lactate, pyruvate and ketone bodies over the cell membrane has been extensively studied in several organs of the body such as muscle tissues and liver (for review see Halestrap and Price, 1999) but it is only recently that these transport processes have been studied in the brain. The monocarboxylates are transported across the cell membrane by diffusional, saturable co-transport with protons in a 1:1 stochiometric ratio. The monocarboxylate transporters (MCTs) form a family of 14 members based on sequence homologies (Halestrap and Meredith, 2004) but only the first four (MCT1 through MCT4) are functionally characterized. Studies have shown that the different transporter subtypes display different affinities for the substrates. For example, MCT2 has the highest affinity for lactate with a Km of ~ 0.7 mM (Garcia et al., 1995; Lin et al., 1998b; Broer et al., 1999) while MCT4 has the lowest affinity with a of Km of ~35 mM (Dimmer et al., 2000; Manning Fox et al., 2000). MCT1 and MCT3 show intermediary affinities of ~ 3.5 mM and 5.8 mM respectively (Garcia et al., 1994; Broer et al., 1997; Yoon et al., 1997) . These different properties have been suggested to account for the distribution of MCT2 in various tissues that take up lactate (eg.liver, Jackson et al., 1997) and the expression of MCT4 in tissues that release large amounts of lactate as a consequence of high glycolytic activity (e.g. fast twitch muscle fibres, Bergersen et al., 2006).

As yet there is limited information about the cellular distribution and function of the MCTs in the CNS. The current information about the distribution of MCTs is based on the expression of mRNA (assessed by in situ hybridization), protein expression (recorded by immunohistochemistry) or both. Available data indicate expression of MCT1, MCT2 and MCT4 in the brain and MCT3 in the basolateral membrane of the retinal pigment epithelium. MCT1 is found throughout the whole rodent brain and is enriched in endothelial cells of blood vessels, astrocytes and ependymocytes (Gerhart et al., 1997; Hanu et al., 2000; Pierre et al. 2000; Baud et al., 2003; Pellerin et al., 2005). MCT2 mRNA is abundant in cortex, hippocampus and the cerebellum of the mouse brain (Koehler-Stec et al., 1998; Pellerin et al., 1998; Debernardi et al.,

2003; Vannucci and Simpson, 2003) and predominantly expressed in neurons (Bergersen et al., 2001, 2005; Pierre et al., 2002). However, there are some contradictory results in the literature (Gerhart et al., 1998; Hanu et al., 2000).

There are as yet few studies that have been conducted on MCT4 in the brain. The existing investigations have mainly assessed its general localization rather than its subcellular distribution. These results suggest an expression in astrocytes of the rat brain (Bergersen et al., 2002; Rafiki et al., 2003). Since MCT4 is a high capacity, low affinity, lactate preferring transporter we hypothesized that it could be involved the clearance of excess lactate from the brain. Thus, the subcellular localisation of MCT4 was investigated using immunogold electron microscopy in CNS (paper III).

Background to methodologies

Rat model of stroke

In the present study, the metabolic recovery of astrocytes and neurons was studied in a rat model of stroke. Focal ischemia was induced by temporarily occluding the middle cerebral artery (MCA) using a modified method originally described by Zea Longa et al., (1989). A thread, coated with poly-l-lysine (Belayev et al., 1996), was introduced through a puncture in the external carotid artery into the internal carotid artery to occlude the MCA. This model allows complete reversal of the ischemia as the thread can be withdrawn after the required ischemic period (more thoroughly described in the Materials and Methods chapter). Two ischemic periods were investigated in both studies as these differ in the size of the infarcts that subsequently develop (Fig. 4). Generally, a 2 h occlusion period results in infarction of the focal area, including the striatum and the overlying cortical focal region. If the ischemic period is extended to 3 h, the damage includes the entire MCA territory, which is similar to that of permanent occlusion. Thus, this model allows us to compare the severity of ischemia that the tissue is exposed to and the infarct that subsequently develops.

FIG. 4. Two representative coronal sections of rat brain after 2 h and 3 h MCA occlusion. The brain slices were stained with 2,3,5-triphenyl 2-H-tetrazolium chloride (TTC) 48 h after occlusion. The white area represents infarcted tissue while pink area is viable tissue.

Methodology to measure astrocytic metabolic activity

The astrocytic oxidative metabolism was selectively evaluated following temporary focal cerebral ischemia *in vivo*, based on intravenously injected [1-^{14}C]acetate and the incorporation of radiolabel into glutamine (Fig. 5). Radiolabeled acetate is converted to acetyl CoA in astrocytes and further metabolized via the tricarboxylic cycle. Glutamine is rapidly labeled in these cells by the conversion of α-ketoglutarate to glutamate and the subsequent action of the synthetic enzyme, GS. The selectivity of this approach is based on the ability of glia but not neurons to take up acetate (Fonnum et al., 1997; Dienel et al. 2001; Waniewski and Martin, 1998, 2004), and on the preferential localization of GS within astrocytes (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979; Petito et al., 1992). A similar approach has been used in some previous investigations of cerebral ischemia in which the products of acetate metabolism were analysed using nuclear magnetic resonance (NMR) (Haberg et al., 1998; Pascual et al., 1998). Only one of these studies (Pascual et al., 1998) has examined a response to reperfusion and this was an investigation of the whole cerebral hemisphere under conditions in which the ischemia was only partially reversed. Therefore, the present study (paper I) aimed at providing information about the recovery of oxidative astrocyte metabolism in astrocytes in different subregions by assessing generation of ^{14}C-glutamine from [1-^{14}C]acetate following focal cerebral ischemia.

Our second approach for evaluating the functional recovery of cells following temporary focal ischemia was to further investigate glucose metabolism in the brain. Particularly, we wanted to assess whether glucose utilization is differentially affected in neurons compared with astrocytes (paper II). The metabolism of this substrate is one centrally important cellular function that has been shown to have long-term alterations following temporary focal cerebral ischemia. In one major study (Belayev et al., 1997), the incorporation of deoxyglucose was substantially decreased at 1 hour after occlusion of the MCA for two hours. This indicates a generally decreased glucose metabolism in the post-ischemic tissue, however, it does not provide information about possible differential responses in the handling of this substrate in various populations of cells.

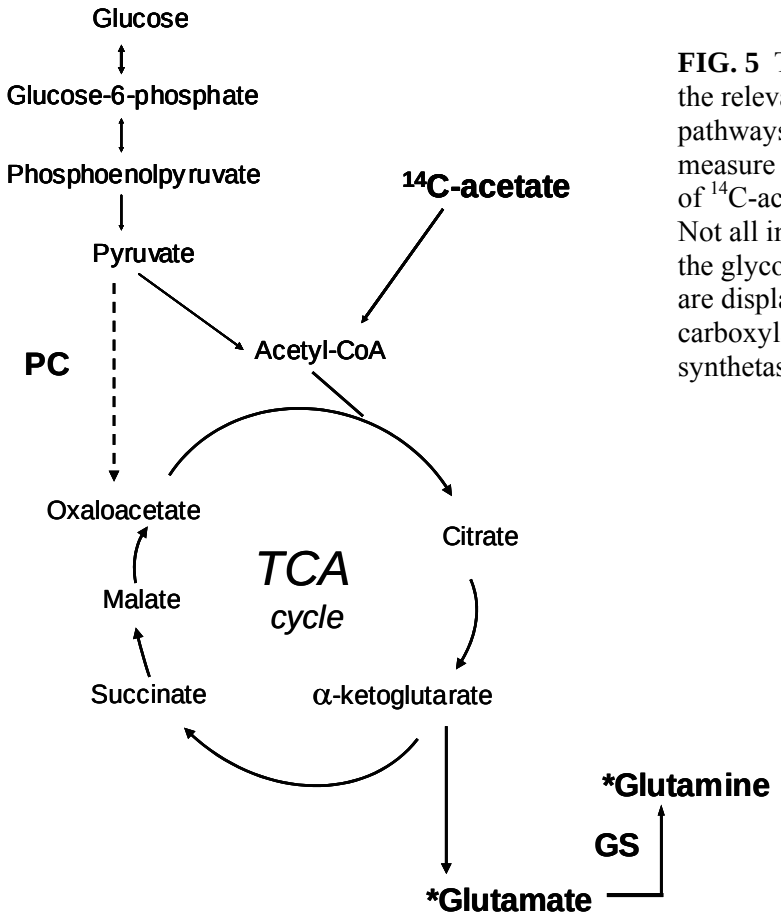

FIG. 5 The figure illustrates the relevant metabolic pathways in astrocytes used to measure oxidative metabolism of ^{14}C-acetate in astrocytes. Not all intermediary steps of the glycolysis or TCA cycle are displayed. PC, Pyruvate carboxylase; GS, glutamine synthetase.

To gain insight into possible differential changes in post-ischemic glucose metabolism in astrocytes and neurons, we assessed the generation of radiolabeled glutamine and glutamate from ^{14}C-glucose during early recirculation following MCA occlusion. Early studies identified that both of these amino acids are rapidly labeled from glucose via glycolysis and the tricarboxylic acid cycle (Lindsay and Bachelard, 1966; Tarkowski and Cremer, 1972). This labeling was shown to result from the activity of two distinct metabolic compartments, one associated with neurons and the other with glia (reviewed by Hertz, 2004). It has been demonstrated that the neurons generate most of the ^{14}C-glutamate that is detected following uptake of ^{14}C-glucose in the brain. Thus, we assessed the neuronal recovery in the post-ischemic tissue through ^{14}C-glutamate content. In contrast, the incorporation of radiolabel into glutamine from glucose does not only arise from astrocyte metabolism but can also be influenced by neuronal function. Astrocytes generate ^{14}C-glutamine from local TCA cycle and subsequent action of GS. ^{14}C-

glutamine can also be produced in astrocytes by the uptake of ^{14}C-glutamate, with neuronal origin. Therefore, it is possible that alterations in glucose oxidation in either cell population or an impairment of the transfer of glutamate from neurons to astrocytes could affect the ^{14}C-glutamine production from ^{14}C-glucose. Studies performed by us and other groups have analysed the differential metabolic changes in astrocytes and neurons which can be exemplified by the effects of fluorocitrate, a selective glial aconitase inhibitor (Fonnum et al., 1997; Willoughby et al., 2003). Intrastriatal injections of fluorocitrate markedly decreased the ^{14}C-glutamine generated within the first ten minutes of ^{14}C-glucose administration. Only small reductions in ^{14}C-glutamate content were observed. This suggests that the majority of the ^{14}C-glutamine arises from astrocyte TCA cycle. Thus, the metabolic recovery of the post-ischemic tissue was assessed by the generation of ^{14}C-glutamine and ^{14}C-glutamate from an intravenous injection of ^{14}C-glucose. The tissue was also investigated for the content of energy-related metabolites as the levels of these can be used as indicators of cell function.

Immunogold cytochemistry

To localize the subcellular expression of MCT4 we employed a postembedding immunogold procedure and electron microscopy. Using this approach, brain tissue from mice was embedded and cut in ultrathin sections whereby epitopes of proteins are exposed at the section surface (Bendayan, 1984, Kellenberger et al., 1987). The identification of the protein of interest, in our case MCT4, is performed by incubating the sections with antibodies raised against a peptide sequence specific for MCT4. In the subsequent step, secondary antibodies coupled to gold particles are used to visualize the bound primary antibodies. It is thus possible to localize the protein and its subcellular expression in the tissue at nanometer resolution. As gold particles can easily be counted it is possible to assess the expression of MCT4 in a semiquantitative manner. The number of gold particles per unit area is directly related to the concentration of the target, as demonstrated by a tailor made calibration system (Ottersen, 1989).

AIMS OF THE STUDIES

The general aim of the study was to evaluate the viability of astrocytes and the recovery of key functions following ischemia in the intact brain. Specifically, we tested the hypothesis that most astrocytes remain viable during the first few hours of focal cerebral ischemia and regain critical metabolic activities during early reperfusion.

SPECIFIC AIMS

I. To assess the recovery of oxidative metabolism in astrocytes based on the generation of ^{14}C-glutamine from [1-^{14}C]acetate during early reperfusion (1 and 4 h) following 2 or 3 h focal cerebral ischemia.

II. To further evaluate cellular metabolic responses to ischemia-reperfusion by assessing the generation of ^{14}C-glutamate and ^{14}C-glutamine from [U-^{14}C]glucose. The production of ^{14}C-glutamate provides a measure that primarily reflects oxidative metabolism in neurons whereas ^{14}C-glutamine is generated in astrocytes using glutamate of neuronal and astrocytic origin.

III. To relate changes observed in the metabolism of [1-^{14}C]acetate and [U-^{14}C]glucose to the content of energy-related metabolites in post-ischemic tissue, to the severity of the ischemic insult and to the subsequent damage that develops in the brain.

IV. To determine the subcellular expression of the monocarboxylate transporter MCT4 in the brain (cortex and cerebellum) and retina in order to identify possible routes by which excess lactate can be removed from the CNS.

MATERIALS AND METHODS

In *vivo* model of stroke-MCA occlusion (I and II)

Surgical procedure

The experimental procedures were approved by the Animal Welfare Committee of Flinders University and are consistent with the Code of Practice of the National Health and Medical Research Council (Australia). Male Sprague-Dawley rats (265-295g) were supplied by the Animal Resource Centre (Gilles Plains, South Australia) or bred in-house (School of Medicine, Flinders University) from the same stock. Rats were fasted overnight prior to surgery. In preparation for surgery, they were intubated and ventilated with a mixture of 23% oxygen/ 77% nitrous oxide (vol./vol.) containing 1-1.5% halothane. Body temperature was maintained throughout surgery using a heating lamp connected to a rectal temperature probe. A polyethylene catheter was placed in the right femoral artery for physiological monitoring of blood gases, blood pressure and blood glucose. A second catheter was placed in the femoral vein, externalized to the tail and taped in place for later administration of [1-^{14}C]acetate or [U-^{14}C]glucose.

Reversible focal cerebral ischemia was achieved by occluding the origin of the MCA using the intraluminal filament technique of Zea Longa et al. (1989) with minor modifications (Anderson and Sims, 1999). A monofilament nylon thread (Dynek sutures, Adelaide, Australia) coated with poly-l-lysine (Belayev et al., 1996) was introduced into the right external carotid artery and advanced through the internal carotid artery to occlude the origin of the right MCA. The wounds were closed, infused locally with 0.5% bupivicaine and the volatile anesthetic discontinued. Animals were tested at 2 h for anticlockwise circling as an indicator of successful MCA occlusion. Rats not meeting this criterion were excluded from the study. Body temperature was monitored post-operatively for 6 h or until euthanasia. Most rats developed hyperthermia as reported previously (Anderson and Sims, 1999). When the temperature exceeded 37.8˚C, rats were placed in an insulated cooling box at a temperature of 7-12˚C to limit the magnitude of this response.

For reversal of ischemia, rats were briefly re-anesthetized with 2 to 2.5% halothane in 33% oxygen / 67% nitrous oxide administered via a face mask and the intraluminal thread withdrawn. Three sham-treated rats underwent the same surgical procedure except that the thread was inserted only a short distance and therefore did not occlude the MCA. Reperfusion was mimicked by withdrawing the thread 2 h later. The rats were injected with radiolabeled acetate or glucose and killed for analysis of metabolic products after a further hour.

Comments

The intraluminal thread model of MCA occlusion is the most widely used model to study pathophysiolgy and therapeutic approaches in permanent and transient focal cerebral ischemia. The model is minimally invasive, not requiring craniotomy, and allows reperfusion. The focal ischemic model was first established by Koizumi et al. (1986) and modified by Zea Longa et al. (1989) to achieve a more reproducible pattern of damage. Nonetheless, there were still reports of considerable variations in the size and distribution of the brain injury. As one means of reducing the variability, we adopted a method developed by Belayev et al. (1997) who coated the threads with poly-l-lysine, a polycationic polymerized amino acid, that increases the adhesive forces around the suture.

The extent of damage produced by MCA occlusion can differ substantially between rat strains and even between the same strain from different sources (Oliff et al., 1996; Duverger and Mackenzie, 1998). In our study, we used male Sprague Dawley rats in all of our experiments. In these animals, a 3 h occlusion period induced a reproducible pattern of damage, generally including all of the MCA territory. A 2 h period generally resulted in infarction in the striatum but more variation in the size of the damage in the cortical focal region.

Consistent with previous reports using the thread occlusion method, most rats developed hyperthermia during the ischemic period (Zhao et al., 1994; Memezawa et al., 1995; Oliff et al., 1996). To minimize the magnitude of this response, rats were placed in a cooling chamber, but nonetheless, most animals showed elevated temperatures. It has been speculated by Zhao et al. (1994) and Memezawa et al. (1995) that the increased temperatures may be caused by hypothalamic ischemia.

Assessment of tissue damage

Tissue damage was assessed in parallel with the metabolic studies to ensure a reproducible pattern of infarction. Rats were decapitated 48 hours after occlusion, the brains removed from the skulls, placed in a perspex brain cutting template and the forebrain cut into 1.5 mm thick coronal sections using a razor blade. The slices were incubated in 3% TTC (in 0.9% NaCl and 20 mM Tris-HCl, pH 7.4) in darkness for 20 min. The slices were then placed in fixative (0.5% glutaraldehyde, 4% formaldehyde in 0.1 M phosphate buffer) and were subsequently digitally scanned.

Preparation of tissue extracts from brain subregions (I and II)

After the required period of recirculation, rats were injected intravenously with 400 µl of 60 µCi [1-^{14}C]acetate or [U-^{14}C]glucose (45-60 mCi/ mmol and 302 mCi / mmol respectively, NEN, Boston, USA) disssolved in 0.9% NaCl and 0.3 mM sodium acetate or 0.3 mM glucose respectively. Rats were decapitated 5 min after the injection. The brains were rapidly removed within 90 s, transferred into a perspex brain mould and frozen in liquid nitrogen. A 3 mm coronal section extending caudally from the rostral limit of the striatum was dissected from the frozen brain. Tissue regions were defined based on a previous study (Anderson and Sims, 1999) and were dissected from the frozen section in a cold box (-10˚C). These regions are illustrated in Fig. 6. In the hemisphere subjected to MCA occlusion, samples were obtained from the striatum and a part of the cerebral cortex ("cortical focal tissue"), regions subjected to severe ischemia. Adjacent perifocal tissue, from an area of the cortex subjected to more moderate ischemia, was also sampled. Corresponding tissue regions were also obtained from the contralateral hemisphere to provide comparisons of the metabolism in tissue that had not been ischemic.

Each tissue sample was added to 200 µl of ice-cold 0.05 M $HClO_4$ and sonicated for 20 s. Homoserine was added (20 µl, 5 mM) as an internal standard. A 20 µl aliquot was removed to determine total tissue radioactivity. The sample was centrifuged (15,000g for 5 min at 4˚C) and the supernatant placed on ice. The pellet was re-extracted using 100 µl 0.05 M $HClO_4$ and re-centrifuged. The supernatants from the two centrifugations were pooled and freeze dried.

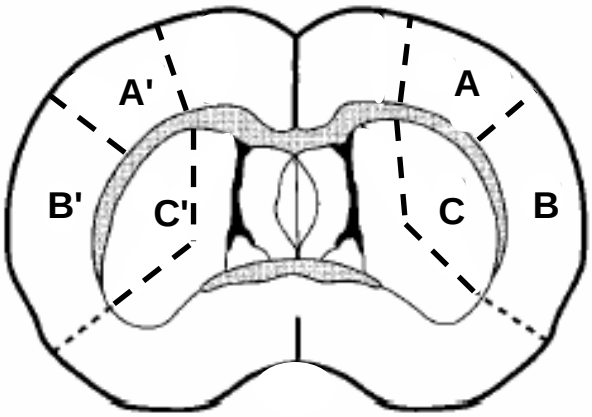

FIG. 6
The figure illustrates the areas dissected out for the analysis of glucose/ acetate metabolism and metabolite studies.
A Cortical perifocal region
B Cortical focal region
C Striatum. Similar regions were obtained from the non-ischemic contralateral side and used as comparison (**A'**, **B**' and **C'**).

The samples were stored at -80˚C and analyzed within two weeks. The pellets from the extraction were solubilized using 1 ml 2 M NaOH and protein determined by the method of Lowry et al. (1951).

Comments

In earlier studies, the maximum specific activity of glutamine was seen at 5 min after intravenous injection of [1-^{14}C]acetate and then declined slowly with a half life of more than an hour (Berl and Frigyesi, 1969). We performed some preliminary studies where it was confirmed that the maximum specific labeling of glutamine was produced at approximately 5 min after injection, with similar values seen at 10 min. We decided to routinely decapitate the rats at 5 min after the injections of radiolabel in our subsequent investigations. This time period provided a balance between incorporating sufficient radiolabel to allow detection and producing a measure that was likely to be sensitive to impairments of metabolism. For the ^{14}C-glucose study, the same time point was used. This 5 min was previously reported to fall early within the period during which there is net generation of radiolabelled amino acids (Cremer, 1970). This ensured that the observed changes largely resulted from alterations in the pathways leading to the incorporation of radiolabel into the amino acids, rather than the subsequent turnover of these metabolites.

To assist with rapid freezing of the brain, a mould was developed for the brain that tolerated liquid nitrogen and provided a template for cutting and navigating to the regions of interest. Thicker brain slices were cut from the frozen tissue compared to those used for TTC

investigations as this avoided cracking. These thicker slices also provided tissue from regions of interest that incorporated sufficient radiolabelled glutamate and glutamine for convenient detection.

A reliable method to extract amino acids from the brain tissue was developed. It was important to obtain high recovery of glutamine and glutamate from the tissue in order to achieve reproducible and reliable results. Initially, we assessed the recovery of the extraction by adding ^{14}C-glutamate to the tissue samples and measured the recovery of the radiolabel. This procedure was also repeated with ^{14}C-glutamine. In parallel with these studies, the recovery of homoserine was assessed and established as a good internal standard to measure recovery. Thus, for later extraction, homoserine was added to each extraction allowing corrections to be made for any losses. Also, the recovery of glutamine and glutamate was found to be improved if the extraction procedure was repeated. This approach was used in subsequent investigations.

A smaller volume was needed for the separation by HPLC than that generated from the initial extraction procedure. Thus, the samples were freeze dried and re-suspended in a small volume of phosphate buffer. This treatment neutralized the sample, which was necessary for the derivatization with o-phthaldialdehyde (OPA) (Tcherkas and Denisenko, 2001).

Measurement of radiolabel incorporation into glutamine and glutamate

Amino acids were derivatized with OPA prior to separation by HPLC and electrochemical detection (EC) using a modification of the method of (Donzanti and Yamamoto, 1988). Prior to derivitization, the freeze dried tissue extracts were dissolved in 70 µl phosphate buffer (0.5 M, pH 7.4) and filtered using 0.45 µm hydrophilic polyproprylene filters (GHP Nanosep MF, Pall Life Sciences, USA). The derivatization reagent was prepared by dissolving 5.4 mg OPA in 200 µl methanol followed by the addition of 2 µl 2-mercaptoethanol and 1.8 ml 0.1 M sodium tetraborate. This reagent was prepared daily and kept sealed in darkness. For determination of radiolabel incorporation, 50 µl of the sample was mixed with 75 µl of OPA reagent and injected after approximately 75 s via a Waters 712 Wispy autosampler (Waters, Milford, Mass., USA).

Separation was achieved using a 100 x 4.6 mm C-18 reversed phase column (3 μm Exsil, SGE Australia) with a guard column (3 μm, SGE Australia). The mobile phase for isocratic elution consisted of 0.1 M Na_2HPO_4, 0.13 mM Na_2EDTA in 28% methanol (pH 6.4) delivered at 0.8 ml/ min using an LC1100 HPLC pump (ICI Instruments, Melbourne, Australia). Derivatized amino acids were detected electrochemically (BAS LC-4B amperometric detector, BAS, West Lafayette, IN, USA) at an applied potential of 600 mV. Samples of eluate were collected at 1 min intervals for 15 min. Fractions corresponding to the glutamine and glutamate peaks were added to 9 ml Readysafe scintillation fluid (Beckman Coulter, Fullerton, CA, USA) and 1 ml water**.** The incorporated radioactivity was measured using a Beckman LS3801 scintillation counter.

The peak electrochemical response of the amino acids in these extracts was too large to allow accurate assessment of the amino acid concentrations. Thus, a second 1 to 2 μl aliquot of the extract was sampled and diluted with phosphate buffer (0.5 M, pH 7.4) to a final volume of 30 μl. This diluted extract was derivatized (45 μl OPA reagent) and subjected to HPLC separation to allow tissue contents of the amino acids to be calculated. Standard mixtures of amino acids were prepared daily from 1 mM stock solutions and treated for analysis as for the tissue extracts. The content of amino acids in the effluent from the HPLC was calculated from comparisons with standards that were analyzed on the same day. The tissue content of amino acids and the incorporation of radioactivity into these amino acids were corrected for recovery of the internal standard, homoserine.

Comments

Preliminary studies established the conditions necessary to separate glutamine and glutamate from brain tissue samples using isocratic HPLC with EC. In order to separate and detect the amino acids, they were pre-derivatised using OPA in the presence of a thiol reducing agent (2-mercaptoethanol). HPLC following OPA derivatization has become a common means of separating amino acids because of the simplicity, speed and sensitivity that can be achieved (Zielke, 1985; Fekkes, 1996; Molnar-Perl, 2003). However, the derivatized products may exhibit variable stability and degrade with time, which might be partially due to excess OPA in the reaction mixture (Stobaugh et al., 1983; Molnar-Perl, 2001). Another study from Tcherkas and Denisenko (2001) showed that glutamine and glutamate were stable for up to 60 min following

the derivatization. To avoid deterioration of the derivatized products with time, we analyzed the samples within 5 min after adding o-phaldialdehyde/ 2-mercaptoethanol. Using this protocol, we did not experience instability in the derivatized amino acids.

We performed some initial studies to determine the recovery of the amino acids after HPLC. The quantity and concentration of OPA/2-mercaptoethanol was optimized. The conditions for optimal separation and recovery were determined by adding ^{14}C-glutamine/ ^{14}C-glutamate to standard amino acid stock solutions or extracts from brain tissue. Fractions were collected and efficiency of separation was determined. Under the optimal conditions developed, derivatized glutamine and glutamate was separated with a recovery of 80-84% for both tissue and amino acid stock solutions.

Content of energy related metabolites (II)

To measure metabolites in brain subregions exposed to ischemia, the brain was frozen in situ after 1 h recirculation as described previously (Ponten et al., 1973). Briefly, rats were anaesthetised with halothane, tracheotomized and ventilated before a longitudinal incision was made in the scalp. A polypropylene funnel was positioned so that Bregma was 2-3 mm from the front edge of the funnel, which was then sutured to the scalp. Liquid nitrogen was poured into the funnel and the freezing front allowed to penetrate the brain for 3 minutes. The funnel was removed and the rat immersed in liquid nitrogen until completely frozen. Tissue was stored at –80˚C until processed.

Brain tissue was dissected and homogenised in a cold box (-30˚C). A coronal section was taken at the level of the striatum using a coarse bladed hacksaw. Tissue samples of 20-30 mg were obtained from brain subregions within the MCA perfusion territory that corresponded to those sampled for determining radiolabel incorporation. Tissue was crushed with a glass rod in 100 µL of methanol acidified with 0.1 M HCl before dispersing in 300 µL of a solution containing 0.3 M $HClO_4$ and 1 mM EDTA. All subsequent procedures were performed at 4^{o}C. Samples were centrifuged at 10,000g for 10 minutes, the supernatant retained and the pellet resuspended in 225 µL 0.3 M $HClO_4$ and 1 mM EDTA for re-extraction. As reported by others (Folbergrova et al., 1992, 1995), the double extraction procedure increased the yield of PCr. The supernatants from

the two extractions were pooled and neutralized using 1.5 M KOH containing 0.3 M imidazole. After centrifugation 10,000g (10 min), the supernatant was stored at -80˚C for subsequent assay. The protein pellets were solubilized using 2 M NaOH for protein determination (Lowry et al., 1951).

Aliquots of 70 µL of neutralised extract were assayed for ATP, ADP, PCr, lactate and glucose according to methods described by Passonneau & Lowry (1993) using a COBAS-FARA automatic spectrophotometric analyzer (Hoffman-La Roche, Basel, Switzerland).

MCT4 expression in retina and brain (III)

Radioactive cDNA probe and Northern blots

The radioactive cDNA probes were constructed and used as explained in paper III. The radioactive signals were visualized with a Phosphor Screen and a Typhoon 9410 imager (Amersham Biosciences). PCR was performed using Q-BioTaq reagents (Q-BIOgene, cat.:EPQBT100, MP Biomedicals, Irvine, USA). Tissue Northerns were commercially obtained from BD Biosciences (rat and mouse MTN Blot).

Cell culture and transfections

Hela cells cells were plated into 9.4 cm^2 wells the day before transfection. Transfection was carried out by complexing DNA into liposomes. The complexes were then diluted in serum-containing medium, cells transfected and media changed after 4 h. Mouse and rat MCT4 (pmMCT4, prMCT4) cDNA plasmid was purchased from Open Biosystems (Genbank accession NM_030696, NM_030834) respectively. The plasmids were propagated by transforming DH5α-competent cells and seeding on LB plates with ampicillin selection. Colonies were picked and expanded overnight in LB-AMP medium at 37°C with agitation. DNA was extracted using a spin column Mini-prep kit (Qiagen) and concentration determined by measuring absorbance with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA).

Cell immunofluorescence

Transfected cells (24 h post-transfection) were washed once with PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and then fixed with 4% formaldehyde in 100

mM NaPi pH 7.4 for 30 min. Cells were permeabilized with PBSX (PBS supplemented with 0.5% Triton X-100) for 15 min, then incubated with antibodies against MCT4 and tubulin, diluted in PBSX, 1:1000 (Alpha Diagnostics, rabbit-anti-rat MCT45-A, raised against amino acids LKAEPEKNGEVVHTPETSV; anti-tubulin, Molecular Probes, Leiden, Netherland) for 2-3 h or overnight, washed 3 times with PBS, incubated for 1 h with secondary antibodies Cy3-goat-anti-rabbit and Alexa Fluor 488-goat-anti-mouse (Jackson Immunoresearch and Molecular Probes, Leiden, Netherland respectively, diluted 1:1000 in PBSX), washed 3 times with PBS and mounted with anti-fade mounting medium containing DAPI. Slides were observed and pictures captured using a Leica LSM5 confocal microscope with appropriate software.

Subcellular fractions, SDS-PAGE and immunoblotting

One 75 cm^2 bottle of transfected cells (70-80% confluency) were trypsinized and cell pellet washed twice in PBS before homogenizing in 300 µl of 10 mM HEPES pH 7.4, 2 mM EDTA, 0.32 M sucrose, protease inhibitor cocktail (Roche) utilizing a pellet pestle (Kontes) and then sonicated for 2 x 30 sec. The resulting lysate was centrifuged 1000 x g 10 min, yielding P1 (cell debris and nuclei) and S1 (post-nuclear supernatant). S1 was then centrifuged 199 000 x g 20 min (Beckman Airfuge, rotor A110 operated at 32 psi) yielding P2 (crude membrane fraction) and S2 (cytosolic fraction). S2 was assayed for total protein using the DC-kit (Bio-Rad) with BSA as a standard. 15 µg of S2 and an equal volume (7 µl) of S1 were loaded on the gel. P2 was resuspended in 20 µl of SDS-loading buffer (Invitrogen) supplemented with 100 mM DTT and 5 µl of this was loaded on the gel.

Gels were 12 % bis-tris from Invitrogen and were run with MOPS-SDS buffer at 200 V for 1 h before blotting onto 0.2 µm PVDF (Bio-Rad) using bis-tris blotting buffer (Invitrogen) supplemented with 20 % methanol at 30 V for 1 h. Blots were blocked for 30 min in 5 % milk powder (Sigma) in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.05 % Tween20) and probed overnight in the cold room with anti-MCT4 diluted to a final concentration of 1 µg/ml in TBS-T. Finally, blots were treated with AP-conjugated secondary antibody and visualized with the ECF substrate (Amersham). Scanning was performed with a Typhoon 9410 and pictures processed with ImageQuantTL.

For the total fractions of brain and retina, male mouse C57BL were sacrificed and decapitated. The regions dissected out and homogenized in 1% SDS, 10 mM sodium phosphate pH 7.4, 5 mM EDTA, 150 mM NaCl, protein inhibitor cocktail (Roche), then centrifuged at 1000g for 10 min and pellet discarded.

Sub-cellular fractionation of cerebrum were performed by dissecting and homogenizing tissue in 0.32 M sucrose, 10 mM HEPES pH 7.4, 5 mM EDTA, protein inhibitor cocktail (Roche), then centrifuged 1000g for 10 min and the supernatant centrifuged at 164 000g for 30 min. The pellet was resuspended in the homogenization-buffer and centrifuged. The final supernatant was decanted and the pellet (crude membrane fraction) was resuspended in 50 mM HEPES, 2 mM EDTA, pH 7.4 and stored at -20°C until use.

Total protein was determined using the DC-kit (Bio-Rad). 30 µg of total protein was loaded onto 10% bis-tris gels (Invitrogen), electrophoresed 200 V 50 min, blotted onto PVDF, blocked in 1% gelatin in TBST, incubated with anti-MCT4 (ADI, 5 µg/ml) in TBST and detected with the ECF kit (Amersham).

Tissue immunofluorescence

Adult male C57BL mice were anesthetized and transcardially perfused with 4% formaldehyde (freshly depolymerized from paraformaldehyde) in 100 mM NaPi, pH 7.4. Tissue was dissected and cryoprotected by immersion in 10%, 20%, 30% sucrose (30 min in each step), 100 mM NaPi, pH 7.4, respectively. Tissue was left in the cold room overnight in the latter solution before freezing on CO_2 pellets. Sixteen µm sections were cut on a Leica cryostat. Sections were rehydrated in PBS, blocked 30 min with PBS-BSA (3% BSA in PBS), incubated with primary antibodies (MCT4, 20 µg/ml, Alpa Diagnostics, USA; mouse-anti-GFAP, Chemicon Int., Temecula, USA, diluted 1:1000; mouse-anti-glutamine synthase (GS), BD Biosciences, Pharmingen, USA, 0.5 µg/ ml; mouse-anti-CD31, BD Biosciences, Pharmingen, USA, 2µg/ ml) for 3 h or overnight in a humidified chamber, washed 3 x 5 min in PBS, incubated with secondary antibodies and nuclear counterstain (TOTO-3 diluted 1:500, Molecular Probes) 1 h, washed 3 x 5 min in PBS and mounted with anti-fade mounting medium containing DAPI. Sections were observed and pictures captured using a Leica LSM5 confocal microscope.

Electron Microsopy

Adult male C57BL mice were anesthetized by an i.p. injection of a mixture of chloral hydrate, magnesium sulphate, and pentobarbital. Retina and brain were fixed by transcardiac perfusion, using 0.1% glutaraldehyde in 100 mM NaPi (pH 7.4). For immunoelectron cytochemistry, small blocks of the eyecup, neocortex and cerebellum were subjected to freeze substitution and infiltrated in Lowicryl as described (Hjelle et al., 1994). Ultrathin sections were processed for immunogold cytochemistry (Matsubara et al., 1996) with MCT4 antibody (10 µg/ 10 ml, MCT45-A, Alpha Diagnostics, USA) followed by gold-conjugated secondary antibody (10 nm particles, BBI Int., UK) for quantitative measurements and statistical analysis. For illustrations, larger gold particles were used for easier visualization (15 nm gold particles, Amersham, UK).

Quantification of gold particles

Digital images were acquired with a commercially available image analysis program (ANALYSIS, Soft Imaging Systems, Münster, Germany). Images of membrane segments were recorded at a nominal magnification of ×46 000. Membrane segments of interest were drawn in the overlay and assigned a type label. Gold particles were attributed to the plasma membrane if they were located within 21 nm of the membrane and detected semiautomatically. Further analyses were done partly in ANALYSIS and partly in the statistical software programme SPSS.

Comments

The transfection experiments in HeLa cells clearly showed that the antibody binds to MCT4. Western blots from the transfected cells revealed distinct bands which were not present in the untransfected control cells. However, mMCT4 showed a lower molecular mass (40 kDa) than predicted (50 kDa). In theory, such a discrepancy could be caused by a premature stop-codon caused by mutations in the plasmid. Sequencing of the plasmid revealed no support to this hypothesis as there was no deviation from the mMCT4 sequence deposited in GenBank. Therefore, the difference in molecular mass remains to be explained. The control cells also demonstrated a weaker band with anti-MCT4. This could be due to endogenous production of MCT4 in the HeLa cells. The fractions of the transfected HeLa cells produced a strong band in the membrane fraction but also a smaller band in the cytosol.

The immunoblots of brain revealed a band at the predicted weight of 50 kDa. When fractions were made of the brain homogenate, a distinct band was seen in the cytosol as well as in the membrane fraction. These fractionation data suggest that the MCT4 antibody crossreacts with a water soluble molecule of unknown identity. If recognized under the conditions of immunocytochemistry, the presence of such a crossreacting molecule could confound the interpretation of our immunocytochemical data. Given this risk, we undertook a careful analysis of the immunogold particle distribution to ensure that the immunoreactive protein (assumed to be identical to MCT4) was localized to the plasma membrane and not in the cytoplasmic matrix.

Statistical analysis

The results are expressed as mean ± SD for the [1-^{14}C]acetate and the [U-^{14}C]glucose studies and as mean ± SEM for the MCT4 study. Paired t-test was used in comparing results from equivalent regions in the occluded and non-occluded hemispheres for the [1-^{14}C]acetate studies. For the [U-^{14}C]glucose studies, values for corresponding regions in the two hemispheres were evaluated by Student's t-test except where indicated otherwise. For the MCT4 study, the means of particle densities were compared between the groups, using the SPSS ANOVA with Scheffe's post hoc test.

RESULTS

Assessment of tissue damage (I)

Tissue infarction produced by 2 h or 3 h of ischemia was assessed at intervals during these studies based on the staining of brain sections with TTC at 48 h after MCA occlusion. Representative sections showing TTC staining at 48 h after either 2 h or 3 h MCA occlusion are presented in Fig. 4. In agreement with previous findings from our group (Anderson and Sims, 1999), 3 h of focal ischemia produced widespread tissue infarction in all rats. This completely encompassed all of the MCA perfusion territory in most animals (7 out of 9). Such damage fully incorporated the cortical perifocal tissue as well as the regions of the striatum and cortex that were sampled as focal tissue for the assessment of acetate metabolism. The shorter 2 h ischemic period produced more variable outcomes. In most rats, there was substantial involvement of the focal tissue in the striatum and cortex but in the perifocal tissue there was either no infarction (2 rats) or partial involvement (4 rats). These observations were reflected in the measurements of infarct volume (expressed as a percentage of the total volume of the tissue region). The infarct volume in the striatum was similar at 48 h after either 2 h (61 ± 39%, n = 6) or 3 h (78 ± 16%, n = 9) of ischemia but in the cortex was substantially less following the shorter ischemic period (2 h ischemia: 30 ± 25%; 3 h ischemia: 55 ± 20%).

Astrocytic oxidative [1-^{14}C]acetate metabolism in the post-ischemic brain (I)

As a measure of the functional recovery of astrocytes during early reperfusion, ^{14}C-acetate metabolism was assessed by measuring the radiolabel incorporated into glutamine. The ^{14}C-glutamine specific activity in the tissue subregions from the two hemispheres following 2 h occlusion and 1 h reperfusion is shown in Fig. 7. The metabolic response to ischemia and reperfusion differed between the three tissue subregions but the overall pattern of responses across the affected brain tissue was very similar following either 2 h or 3 h of focal ischemia.

The largest effect was seen in the striatum (Fig. 7). For all of the rats studied following both of the ischemic insults, ^{14}C-glutamine specific activity in the striatum subjected to occlusion was less than in the corresponding tissue from the contralateral hemisphere.

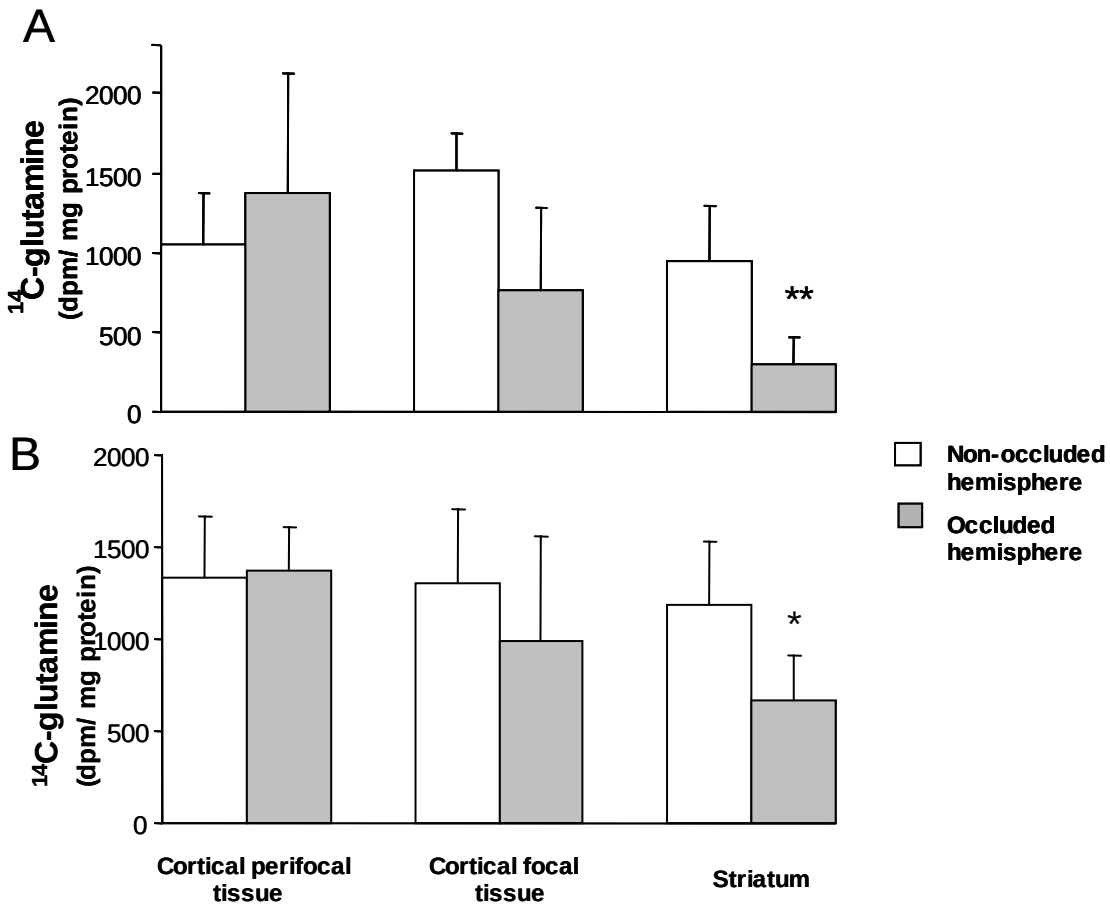

FIG. 7. Incorporation of radiolabel from [1-^{14}C]acetate into glutamine at 1 h of reperfusion following **A** 2 h **B** 3 h MCA occlusion. Values are shown as mean ± SD (n = 6, *p < 0.05, **p < 0.01, paired t-test).

In contrast, the radioactivity in glutamine in the cortical perifocal tissue was similar in the two hemispheres. There was also no significant difference between hemispheres in radiolabel incorporation into glutamine in the focal tissue from the cortex. However, the response was much more variable between animals in this region than in cortical perifocal tissue.

Radiolabel incorporated from [1-^{14}C]acetate into glutamate was also investigated. ^{14}C-glutamate can arise from the astrocytic TCA cycle but also from glutamine released by the astrocytes that is subsequently taken up by neurons and converted to glutamate. Thus, ^{14}C-glutamate content may reflect both astrocyte metabolism and the capability of neurons to take up glutamine and convert it to glutamate. ^{14}C-glutamate labeling was significantly reduced in the focal areas after both 2 and 3 h MCA occlusion with 1 h reperfusion. No significant changes were seen in the cortical perifocal tissue at the same reperfusion and ischemic time points.

To investigate possible further deterioration of astrocytic function at longer reperfusion periods, [1-^{14}C]acetate metabolism was investigated at 4 h of reperfusion following 3 h of ischemia. Interestingly, the relative metabolic activities in the treated and contralateral hemisphere were very similar to that seen at 1 h of reperfusion. In the striatum, there was again a significant decrease in incorporation of radiolabel, with all rats showing lower values for the hemisphere subjected to ischemia compared with tissue from the contralateral hemisphere. There was no significant difference in either of the cortical regions investigated.

Glucose metabolism in the post-ischemic brain (II)

As a further measure of the functional recovery of the post ischemic tissue, the metabolism of [U-^{14}C]glucose was assessed through the incorporation of radiolabel into glutamine and glutamate. The incorporation of radiolabel was assessed at 1 h reperfusion following 2 or 3 h MCA occlusion. The results showed that there were marked reductions in both ^{14}C- glutamate and ^{14}C-glutamine content at 1 h of reperfusion following 3 h of MCA occlusion. Reductions were evident in each of the three regions that were analyzed in the hemisphere ipsilateral to the conclusion, when compared with equivalent samples from the contralateral hemisphere (Fig. 8). Incorporation of radiolabel into both amino acids was severely reduced but ^{14}C- glutamate content showed moderately larger changes in all regions. The decreases appeared to relate to the severity of ischemia during MCA occlusion. The metabolism at 1 h of recirculation following 2 h of ischemia also showed marked reductions in all three regions exposed to ischemia. However, there were some subtle but possibly important differences compared with the response to 3 h of ischemia. The reductions in focal regions in the cortex and striatum were moderately less following the 2 h ischemic period (averaging a 55% decrease for ^{14}C- glutamate and 50% for ^{14}C-glutamine). Interestingly, decreases in the perifocal tissue were similar to those in the focal tissue at this time.

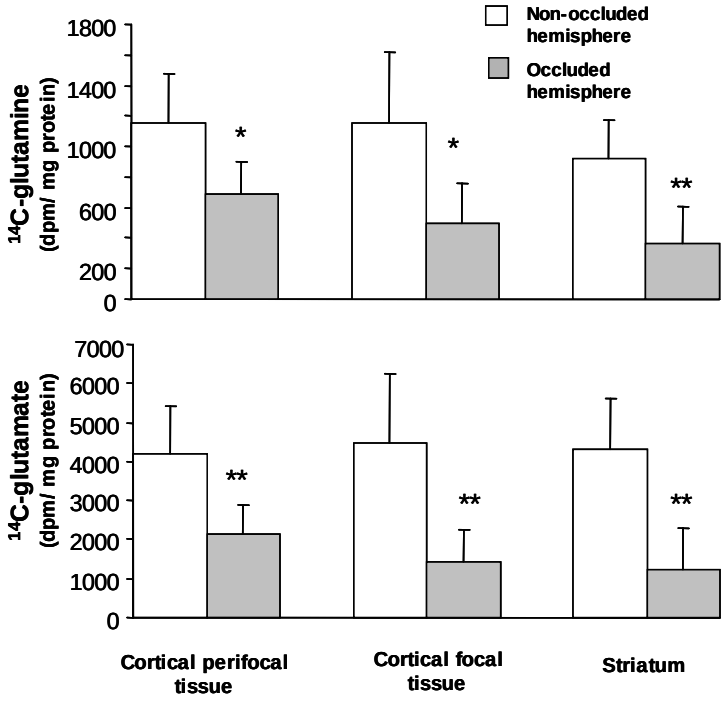

FIG. 8. ^{14}C-glutamate and ^{14}C-glutamine content produced from [U-^{14}C]glucose metabolism, assessed at 1 h reperfusion following 3 h MCA occlusion. The results are shown as means ± SD (*p< 0.05, **p< 0.01, Student's t-test).

Based on detection of these widespread reductions in ^{14}C-amino acid content, radiolabel incorporation into the two amino acids was also measured in cortical tissue from an area outside the MCA perfusion territory in subsequent animals. For this purpose, tissue was sampled from the cerebral cortex overlying the striatum and immediately adjacent to the midline (paramedian cortex). Both of the amino acids showed a similar trend to that of the MCA territory but the difference only achieved statistical significance for the content of ^{14}C-glutamate after 2 h occlusion.

Tissue metabolites at 1 h of recirculation following focal ischemia (II)

To further evaluate the metabolic state of the postischemic tissue and assist with interpretation of the findings from ^{14}C-glucose and ^{14}C-acetate studies, the content of energy related metabolites from regions within the MCA perfusion territory were also assessed in samples from brains frozen *in situ*. The glucose content in brain tissue extracts was similar in samples from the subregions in the hemisphere subjected to temporary MCA occlusion and those from the contralateral hemisphere. This suggests that differences in dilution of the ^{14}C- glucose on entry into the brain were unlikely to be a significant factor influencing the incorporation of radiolabel into the amino acids.

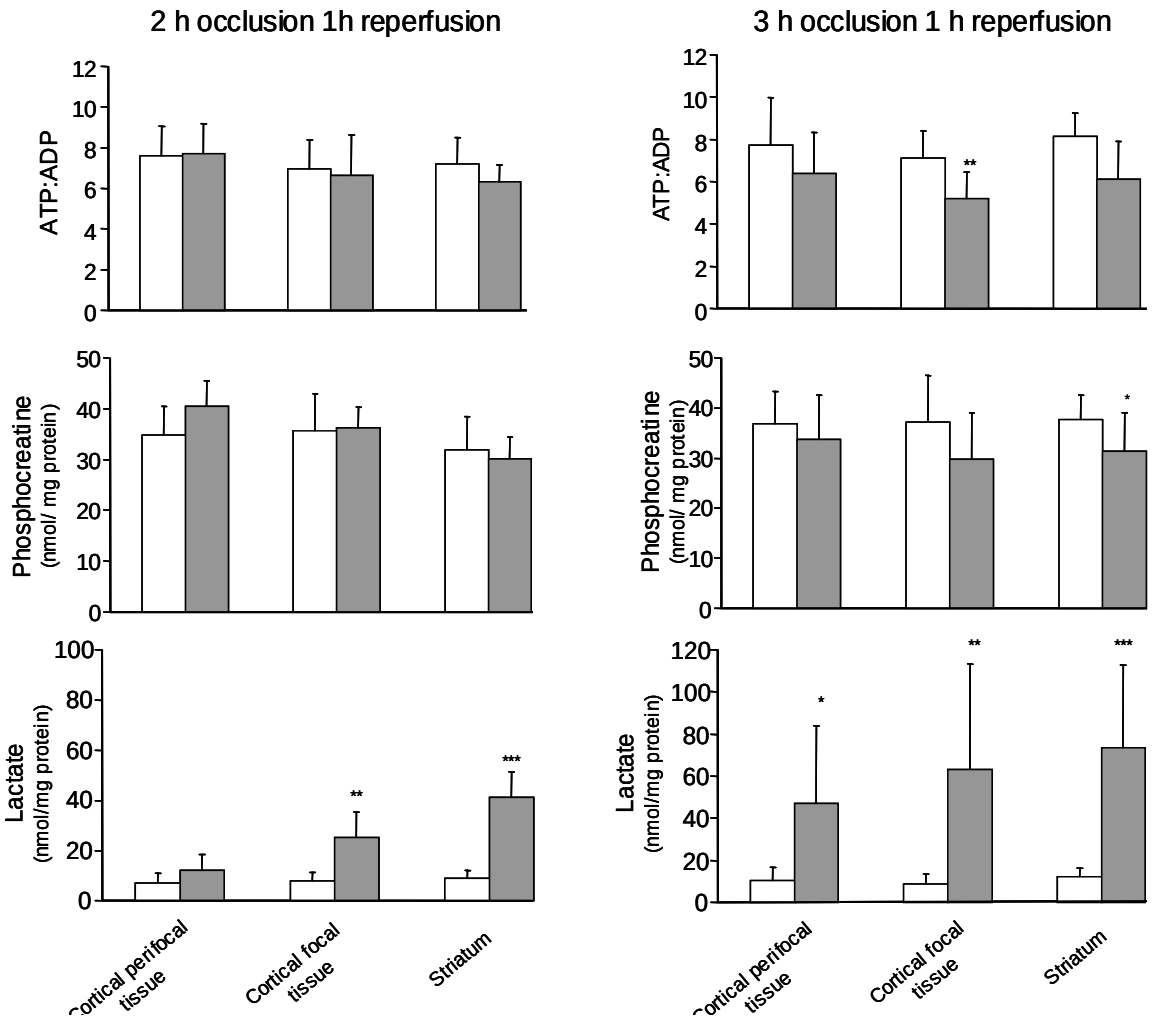

FIG. 9. Metabolite data from post-ischemic tissue following MCA occlusion. ATP:ADP ratio, PCr and lactate content in brain subregions after 1 h of reperfusion following 2 or 3 h occlusion. Values from the occluded hemisphere (grey bars) are compared to those of values from the non-ischemic contralateral hemisphere (white bars) and are shown as mean ± SD (*p< 0.05, **p< 0.01, ***p< 0.001, Student's t-test).

Occlusion of the MCA for 3 h with 1 h reperfusion produced significant decreases in the tissue content of both ATP and ADP in all three affected subregions compared with the corresponding samples from the contralateral hemisphere. Because of the reductions in both nucleotides, the ATP:ADP ratio showed smaller differences and this was statistically significant only for the cortical focal tissue (Fig. 9). These findings suggest that a partial depletion of the total nucleotide pool during MCA occlusion was a likely significant contributor to the decrease in ATP content. Losses of this metabolic pool have been reported previously during cerebral ischemia and recover only slowly on restoration of flow (Selman et al., 1990; Folbergrova et al., 1995).

The extent of preservation of the ATP:ADP ratio suggests that many cells in the tissue remained viable and capable of essentially balancing their energy use and production. This interpretation is supported by measurements of the content of PCr. This metabolite provides a short-term energy reserve and is normally depleted prior to ATP under conditions of high energy demands. A small decrease in PCr following 3 h occlusion was seen in the striatum whilst differences in the cortical regions were not statistically significant. In contrast to the limited changes in energy metabolites, there were very large average increase in lactate content in all three regions from the treated hemisphere, although there was considerable inter-animal variability (Fig. 9). Lactate is the product of anaerobic glucose metabolism and the accumulation of this metabolite provides further evidence of marked alterations in the handling of glucose in the post-ischemic brain.

A similar pattern of change in metabolites was seen in the brain tissue subjected to 2 h MCA occlusion although any changes tended to be smaller than seen during reperfusion following 3 h of ischemia. There were again significant differences in the ATP and ADP content in the focal tissue from the cortex and striatum but no significant difference was detected in the cortical perifocal tissue. Neither the ATP:ADP ratio nor the PCr content were significantly affected by ischemia and reperfusion in any of the three regions in the MCA territory. Lactate content was again substantially increased in both the cortical focal tissue and striatum but these increases were smaller than those observed following 3 h MCA occlusion. No significant increase in lactate was observed in the cortical perifocal tissue, a region that is much less likely to become infarcted following 2 h ischemia.

Supporting investigations (I and II)

Total glutamate and glutamine content in the tissue

The total tissue contents of glutamine and glutamate were measured in the samples to investigate the possible changes in these metabolic pools that might influence the incorporation of radiolabel from either [1-^{14}C]acetate or [U-^{14}C]glucose into glutamate and glutamine. The content of these amino acids after 2 or 3 h occlusion following 1 h reperfusion in the studies of [1-^{14}C]acetate and [U-^{14}C]glucose metabolism were very similar and revealed substantial preservation of both glutamate and glutamine in most regions.

Total radioactivity in tissue samples

The total radioactivity in the unfractionated tissue homogenates prior to extraction of the amino acids was determined to test for possible changes in delivery of the radioactive substrates in the hemisphere that had been subjected to ischemia. During reperfusion following either 2 or 3 h occlusion in the studies involving [1-^{14}C]acetate or [U-^{14}C]glucose, the total radioactivity detected in the tissue homogenate samples was generally similar in the two hemispheres. Thus, these findings suggest that the changes observed in the incorporation of radiolabel into glutamine and glutamate in either of the studies were not likely to have resulted from different availability of substrate in the hemisphere previously subjected to ischemia.

Sham operated rats

To ensure that the results from the changes in [1-^{14}C]acetate or [U-^{14}C]glucose metabolism were not a result of the experimental procedures alone, three rats in each study were sham operated. There were no statistical differences between the right and the left hemisphere in the incorporation of radiolabel into glutamine and glutamate in either of the studies. The amount of radiolabel incorporated into the two amino acids was also similar to that of the control hemisphere of rats subjected to MCA occlusion. Therefore, the results seen in the [1-^{14}C]acetate or [U-^{14}C]glucose studies were not due to the experimental procedure alone.

Diffusion of radiolabel (I)

To test whether the apparent recovery of ^{14}C-glutamine production could be influenced by diffusion of this substance from adjacent tissue or the circulation, we investigated ^{14}C-glutamine content in tissue that had progressed to infarction. ^{14}C-acetate metabolism was assessed in 2 rats at 48 h after MCA occlusion. Most of the ^{14}C-glutamine was lost in the three regions with an average decrease of 84% ± 11%. These findings suggest that almost all of the ^{14}C-glutamine detected was a result of local astrocytic metabolism.

Expression of MCT4 in retina and brain (III)

Northern blots

MCT4 expression was investigated at the mRNA level using a probe generated from an mMCT4 cDNA clone. The probe was directed against a sequence corresponding to exon 5. The Northern blots revealed a rather weak signal in the mouse brain relative to the signal from muscle. This finding is consistent with published data on MCT4 mRNA expression in human tissues (Price et al 1998). The Northern blots were standardized to β-actin, the concentration of which might vary somewhat between tissues. A probe to GFAP gave a strong signal in brain tissue, confirming that the blots contained appropriate amounts of brain derived (and astrocyte derived) mRNA. On this background we conclude that the rodent brain contains MCT4 mRNA, but that the expression level is substantially lower than in muscle.

Plasmid transfection

In order to validate the selectivity of the MCT4 antibody, HeLa-cells were transfected with a plasmid encoding the mouse MCT4 protein (pmMCT4). The transfected cells were strongly immunoreactive for MCT4. Western blots revealed a distinct band at 40 kDa in the transfected cells, which were not present in the untransfected control cells. A distinct band was also revealed in untransfected cells which could be due to an endogenous production of MCT4 in HeLa cells (see comments in Materials and Methods). As expected, the MCT4 signal was much stronger in the crude membrane fraction than in the cytosolic fraction. These data confirm that the MCT4 antibodies recognize the MCT4 protein.

Western blots of brain and retina

A distinct band at 50 kDa was seen from brain homogenates (cerebrum and cerebellum). Substantial amount of protein had to be loaded onto the gel in order to get a distinct band. This again confirms that there is a low expression of MCT4 in the brain. However, when fractions were made of the cerebral homogenate, a prominent band was revealed in the cytosol fraction as well as in the membrane fractions. We have currently no good explanation of the cytosol band (see Material and Methods).

Immunofluorescence and immunogold electron microscopy

Double immunofluorescence labeling for MCT4 and GFAP indicated that MCT4 is concentrated in astrocytes in the neocortex. The MCT4 immunofluorescence signal was particularly distinct around microvessels and at the subpial and subvitreal surfaces. Glial cells in the retina and cerebellum also showed immunoreactivity for MCT4. The perivascular MCT4 labeling was weaker in the cerebellum than in the retina and neocortex.

By postembedding immunogold electron microscopy it was confirmed that MCT4 is enriched in glial endfoot membranes facing capillaries (Fig. 10A), pia (Fig. 10B), and corpus vitreum (not shown). No immunogold labeling was found in pre-absorbtion control. Scattered gold particles were also found over astrocyte membrane domains in the neuropil (not shown). Analysis of the gold particle distribution along an axis perpendicular to the astrocyte endfoot membrane showed a distinct peak over the membrane and a symmetric decline in gold particle density on either side. Background levels of gold particle density was reached at ~20 nm off the membrane, corresponding to the dimension of the antibodies that are interposed between epitope and gold particle (Matsubara et al., 1996). In the cerebellum and retina, glial membranes facing neuropil revealed minor peaks of MCT4 immunogold labeling (not shown). No such peak could be identified in the cerebral cortex. The density of gold particles was quantitatively assessed and attributed to the plasma membrane if they were located within 21 nm of the membrane. Using this criterion, it was confirmed that perivascular endfeet membranes were significantly more strongly labeled than other glial membranes.

The number of gold particles was quantitively assessed in the endfeet membranes abutting on endothelial cells and domains abutting on pericytes. The quantitative analysis revealed regional differences in regard to the intensity of the endfoot labeling for MCT4. Specifically, retinal glial endfeet displayed a linear density of MCT4 immunogold particles that was twice as high as that of cerebellar glial endfeet. Intermediate values were recorded for endfeet in the neocortex.

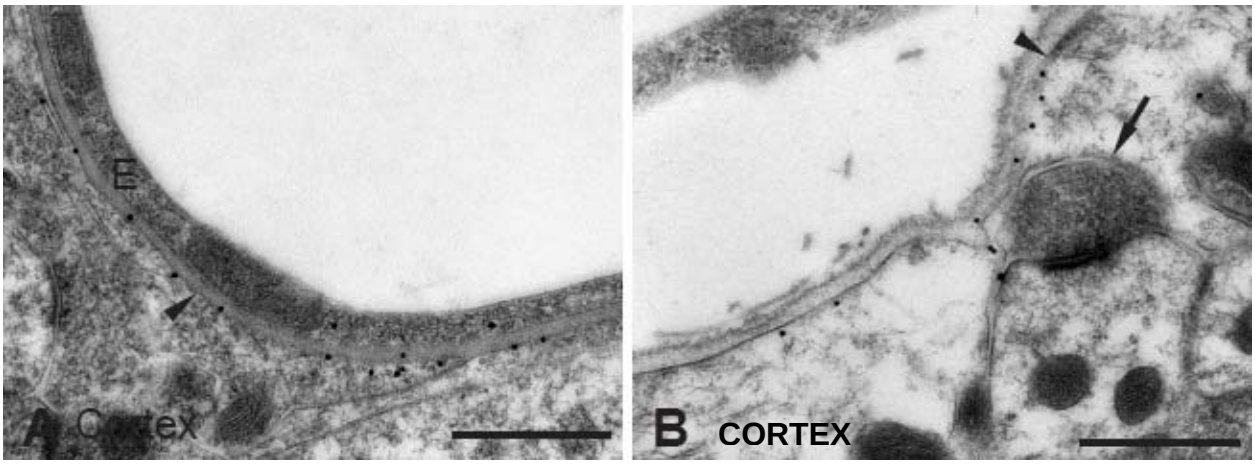

Fig. 10 Enrichment of MCT4 immunogold labeling in astrocyte endfeet
Postembedding immunogold electron microscopy revealed an enrichment of MCT4 in astrocyte endfoot membranes facing capillaries (A) and pia (B) in neocortex (arrowheads). Low expression of MCT4 immunogold labeling was seen in neuropil (arrow). E, endothelium. Size marker, 500 nm.

DISCUSSION

The current study has shown that the astrocytes in the cortical regions regain their oxidative capacity following focal cerebral ischemia (paper I). The results imply that the astrocytes in these regions are viable and capable of complex metabolic activity several hours following ischemia. This may suggest that astrocytes are a promising target for future pharmacological interventions. Astrocytes in the striatum were apparently more sensitive to the ischemic- reperfusion injury since there was significant reductions in glutamine labeling in this region. In contrast to the acetate study, there were substantial alterations in the metabolism of [U-^{14}C]glucose at 1 h reperfusion in all regions that had been exposed to ischemia (paper II). The generation of ^{14}C-glutamate, which is almost exclusively neuronal, was severely decreased. Reductions in ^{14}C-glutamine were also observed. These alterations in [U-^{14}C]glucose metabolism were not due primarily to loss of cell viability as most regions showed near normal ATP:ADP ratios and PCr content. The changes in amino acid labeling were not closely related to the infarction that subsequently develops, indicating that these changes were not a good predictor of cell death.

Elevated levels of lactate were observed in most of the regions of the post-ischemic tissue and the quantity of this metabolite was related to the subsequent extent of infarction. The most likely explanation for the increased lactate levels is impaired pyruvate oxidation. However, the increased lactate level could also reflect impaired removal. This prompted us to investigate the mechanisms by which excess lactate is cleared from the normal CNS. MCT4 was found to be concentrated at the endfeet of astrocytes facing capillaries and pial surface of the brain, as demonstrated by immunogold cytochemistry. The functional characteristics of MCT4 and its dense localization in glial endfeet suggest that it contributes to the clearance of excess lactate from the CNS.

The results from the first paper indicate that astrocytic function, assessed from ^{14}C-acetate metabolism, is substantially different between brain subregions during the first few hours following focal cerebral ischemia. In the striatum, this metabolic response is consistently impaired under each of the conditions investigated. In contrast, ^{14}C-acetate metabolism was generally preserved in the cortical perifocal tissue that was subjected to more moderate ischemia.

The focal tissue within the cortex showed more variability in response between animals but metabolic activity was also largely preserved in more than half of the rats investigated. Thus, in these animals, most astrocytes in the cortex apparently remained viable following the extended periods of ischemia and retained the capacity to oxidatively metabolize acetate via the tricarboxylic acid cycle. ^{14}C-acetate metabolism was also investigated at a later time point, at 4 h reperfusion. No further deterioration of ^{14}C-acetate metabolism was observed at this stage, compared with the situation at 1 h of reperfusion.

Results from a very recent investigation from Håberg et al. (2006) support our findings. In this study, the metabolic recovery of astrocytes and neurons was assessed using NMR of tissue extracts from brain in rats subjected to 2 h MCA occlusion and 2 h reperfusion. The rats received an intravenous bolus injection of [1-^{13}C]glucose and [1-^{13}C]acetate. Similarly to our investigations, the isotope incorporated into glutamine and glutamate was assessed although their labeling period was longer (15 min). Their results showed a significant reduction in the incorporation of radiolabel from acetate into glutamine in the ischemic core and an increase in the penumbra. This supports our findings that the astrocytes in the cortical perifocal area (penumbra) are capable of oxidative metabolic activity several hours after the occlusion. As they did not distinguish between striatum and overlying cortex of the focal tissue, it is possible that the decreased ^{13}C-glutamine that they observed reflected primarily the changes in the striatal part of the ischemic core.

Our results also demonstrated significant reductions in the incorporation of radiolabel from ^{14}C-acetate into glutamate in the striatum and cortical focal tissue. Such reductions were also reported by Håberg et al. (2006) but they also observed reductions in the penumbra. The decreased ^{14}C-glutamate indicate an impairment in either the subsequent uptake of glutamine in the neurons or reduced activity of the enzyme phosphate activated glutaminase (PAG) that convert glutamine to glutamate in these cells. Such a change would be expected to be more readily detected with a longer period of isotopic labeling following injection of labeled acetate, as was the case in the study by Håberg et al. (2006).

The vulnerability of striatal astrocytes to metabolic impairment is consistent with other evidence for rapid progression towards cell dysfunction and death following ischemia. Other metabolic properties, including mitochondrial respiratory capacity (Anderson and Sims, 1999) and mitochondrial glutathione content (Anderson and Sims, 2002), have also been found to be affected to a greater degree in the striatum than in the cortex during early recirculation following MCA occlusion. Pronounced changes were also identified in some cell-specific proteins in the striatum compared with the cerebral cortex during early recirculation following 3 h of MCA occlusion (Lee et al., 2003). One possible explanation for the differential response to ischemia is subtle regional differences in the reduction in blood flow. However, previous investigations indicate that the cerebral cortex and striatum differ only marginally when it comes to the extent of blood flow reduction during MCA occlusion (Bolander et al., 1989; Nagasawa and Kogure, 1989; Belayev et al., 1997). Alternatively, the greater vulnerability of the striatum could be due to regional differences in the properties of astrocytes. In support of this idea, astrocytes derived from the striatum and grown in culture show larger vulnerability to OGD compared to that of astrocytes from the cortex (Xu et al., 2001).

There was little difference in the pattern of ^{14}C-acetate metabolism following either 2 h or 3 h of ischemia. There were no obvious relationship between the metabolic changes in tissue regions and the pattern of tissue infarction that subsequently developed. This is most obvious for the cortical perifocal tissue. Tissue infarction, which involves the death of all cells including astrocytes, is usually initiated throughout this region following 3 h of ischemia but is not induced or is incomplete following 2 h of ischemia. However, the acetate astrocytic response was similarly preserved following both ischemic insults. Thus, the deterioration and death of these cells following 3 h of ischemia is clearly markedly delayed in this region. Both insults typically produce infarcts encompassing focal tissue within the striatum and the cortex. The difference in response between these two subregions further suggests that early changes in this aspect of astrocytic oxidative metabolism are not essential for subsequent progression to tissue infarction.

Whilst the findings of the present study suggest good preservation of the oxidative capacity of cortical astrocytes, it does not necessarily indicate that the metabolism of glucose will be similarly preserved in these cells. In fact, the results from the second paper suggest marked

alterations of cellular glucose utilization in all regions in the post-ischemic brain. The large reductions in the content of ^{14}C-glutamate indicate impaired or reduced neuronal glucose metabolism. Decreases in glucose consumption following temporary focal ischemia have been reported in a study using deoxyglucose (Belayev et al., 1997). This technique does not distinguish between the contributions of the different cell populations. However, the findings suggest that a depression of overall glucose metabolism was probably a major contributor to the decreased labeling of glutamate and glutamine that we observed.

Significant reductions in the incorporation of radiolabel from ^{14}C-glucose into glutamine were also recorded. However, the interpretation of these data is somewhat more complicated. ^{14}C-glutamine can arise from astrocytic TCA cycle but it can also be generated from glutamate produced in neurons. Thus, impairment in the neuronal metabolism is likely to contribute to the decrease in ^{14}C-glutamine content. The sources of glutamate for glutamine production have been studied by others, but different results have been reported depending on the labeling infusion times and whether the animals have been anaesthetized or not. NMR studies of isotope incorporation into individual carbons of glutamine during extended infusions of ^{13}C-glucose and other substrates have shown that glutamate produced in neurons is the major precursor for glutamine synthesis, with oxidative metabolism of glucose in astrocytes contributing up to approximately one-third of the total (Aureli et al. 1997; Gruetter et al. 2001; Öz et al. 2004). Merle et al. (2002) identified differences in the relative labeling of individual carbons in glutamine over time and suggested that the contribution from astrocytic glucose oxidation was much larger at short periods after injection of the labelled glucose. This finding is potentially relevant to the short (5 min) isotopic-labeling period used by us. However, in contrast to our investigations, Merle et al. (2002) examined anesthetized rats. A more recent study in conscious rats did not show similar changes in the relative labeling of glutamine carbons over time and provided findings consistent with a predominant neuronal contribution of ^{14}C-glutamate to ^{14}C-glutamine generation at all time points (Öz et al. 2004). Thus, the most likely interpretation of our results is that the decrease in ^{14}C-glutamine accumulation largely resulted from the reduced supply of ^{14}C-glutamate from neurons to the astrocytes. The recent study by Håberg et al. (2006), using NMR, also demonstrated large reductions in glutamate and glutamine labeling from ^{13}C-glucose in both the focal ischemic region and the penumbra, which provides support for our

findings. Again, this method does not allow identification of the origin of the glutamate used for glutamine synthesis.

The alterations in ^{14}C-glucose metabolism were again apparently not closely related to subsequent progression to tissue infarction in these rats. In the present study, decreases in ^{14}C-glucose metabolism following 2 h of ischemia were at least as large in the cortical perifocal tissue, a region that does not develop tissue infarction following this ischemic period, as in the focal tissue from both the striatum and cortex. A region outside the MCA territory also showed decreases ^{14}C-glucose metabolism, particularly affecting ^{14}C-glutamate generation. Similarly, it has been demonstrated that there is a decrease in glucose metabolism which extends beyond the area that eventually undergoes infarction in a cat MCA model (Heiss et al., 1997 a, b). Intermittent waves of reversible depolarizations that resemble spreading depression commonly develop in perifocal regions during focal ischemia (Koroleva and Bures, 1996; Nedergaard and Hansen, 1993; Nedergaard, 1996). Cellular changes associated with the migration of these depolarizations might provide a trigger for the development of metabolic responses in tissue distant from the primary insult. It has been suggested that astrocytes may contribute to spreading depression by triggering secondary depolarizations in neurons (Walz, 1997; Martins-Ferreira et al., 2000; Lian and Stringer, 2004).

Interestingly, the widespread changes in ^{14}C-glucose metabolism at 1 h of recirculation following 2 h of ischemia were not associated with significant alterations in either the ATP:ADP ratio or the PCr content. Preservation of these critical metabolites indicates that extensive cell damage or death was not a significant contributor to the changes seen at this time. The reduced glucose metabolism with the simultaneous preservation of ATP:ADP ratio and PCr suggests that energy demands are reduced in the post-ischemic brain, perhaps as a “stress” response to the ischemia or reperfusion. Decreases in cerebral glucose use have been reported previously following hypoglycemia (Abdul-Rahman and Siesjo, 1980), transient global ischemia with reperfusion (Pulsinelli et al., 1982; Choki et al., 1983;) and several forms of traumatic injury (Pappius, 1991; Yoshino et al., 1992) consistent with this being a common response of brain cells to pathological conditions. Synaptic activity is apparently depressed for long periods in response to temporary

MCA occlusion (Heiss and Rosner, 1983; Bolay and Dalkara, 1998; Bolay et al., 2002), providing one possible contributor to reduced energy demands under these conditions.

The pattern of changes in incorporation of radiolabel from glucose into the amino acids was largely similar during early recirculation although there were some subtle but potentially important differences between 2 h and 3 h of ischemia. Again, decreases consistent with marked changes in glucose metabolism in astrocytes and neurons were seen, albeit with a tendency for the primarily neuronal ^{14}C-glutamate generation to be more severely affected. Following the longer 3 h period of MCA occlusion, there appeared to be a closer link to the severity of the ischemic insult. The metabolism of ^{14}C-glucose after 2 h ischemia demonstrated large reductions of radiolabeled amino acids in the focal tissue which were greater than in the cortical perifocal tissue from the same brains. Some moderate but significant changes in the ATP:ADP ratio and in PCr were seen in focal regions following 3 h of ischemia together with a large accumulation of lactate in all regions within the MCA perfusion territory. These findings suggest an impairment of oxidative metabolism leading to lactate accumulation and a diminished capacity to maintain ATP production.

Lactate content was increased in the focal tissue regions following 2 h of ischemia but was not significantly altered in cortical perifocal tissue. Thus, the increases in lactate content appeared to relate more closely to the subsequent development of infarction than the changes in ^{14}C-glucose metabolism. Consistent with this conclusion, Håberg et al. (2006) detected synthesis of lactate in striatal tissue but not in the neocortex following 2 h of MCA occlusion and 2 h reperfusion. Earlier studies have also observed increased lactate levels during the first few hours of reperfusion following periods of ischemia that were sufficient to initiate infarct formation (Selman et al., 1990; Folbergrova et al., 1995; Franke et al., 2000). This ongoing elevation of lactate content in the tissue despite restoration of blood flow may suggest a persistent impairment of pyruvate oxidation. The partial pressure of oxygen exceeds control values for several hours in reperfused brain tissue (Folbergrova et al., 1995; Nakai et al., 1997) indicating that oxygen availability is unlikely to limit the oxidative metabolism. Partial inactivation or degradation of the pyruvate dehydrogenase (PDH) complex has been observed during reperfusion in tissue regions containing neurons selectively vulnerable to transient global ischemia (Cardell et al., 1989;

Zaidan and Sims, 1993; Bogaert et al., 1994, 2000; Fukuchi et al., 1998; Zaidan et al., 1998). Activity of the PDH complex can be impaired by increases in reactive oxygen species (Tabatabaie et al., 1996) providing a possible explanation for reduced pyruvate oxidation in the post-ischemic brain. Recent studies have also demonstrated that resuscitation with 100% oxygen, which is standard clinical practice following cardiac arrest, results in reduced PDH activity in the hippocampus. The activity of this enzyme was found to be improved with normoxic resuscitation, suggesting that PDH is affected by ROS *in vivo* (Richards et al., 2006; Vereczki et al., 2006). Thus, it is likely that a decrease in pyruvate oxidation promoted lactate production, diverting radiolabel from glucose into lactate and contributing to the reduced generation of radiolabeled amino acids.

The elevated lactate observed following ischemia, particularly after the longer ischemic insult, is likely to reflect direct impairment of pyruvate oxidation. A reduced clearance of lactate from the brain could also contribute to lactate accumulation. The mechanisms by which excess lactate can be removed from the CNS have not been clearly elucidated. This prompted us to investigate the distribution of the lactate preferring transporter MCT4 (paper III). MCT4 has previously been demonstrated to remove large amounts of lactate and protons from glycolytically active tissues (Bonen et al., 2000; Dimmer et al., 2000; Manning Fox et al., 2000) but its subcellular distribution in the CNS is unknown. Our investigations showed that this low affinity, high capacity, lactate preferring transporter was primarily and densly expressed in the glial endfeet facing blood vessels in retina, cortex and cerebellum. MCT4 was also expressed in the glial endfeet facing the pial surface of the brain. Astrocytes and Müller cells have been proposed to increase their glycolytic rates in response to neuronal activity and glutamate release, producing and releasing lactate for neuronal use (Poitry et al., 2000; Pellerin and Magistretti, 2004). However, it is currently unknown to what extent the neurons are dependent on lactate as a metabolite for energy production. It is plausible that glial cells during neuronal activity or following a CNS injury increase glycolysis and become net producers of lactate. A small net release of lactate into the blood stream has been observed from the awake resting brain (Juhlin-Dannfelt, 1977). Larger amounts are released during generalized sensory stimulation (Dienel and Cruz, 2003), spreading cortical depression (Cruz et al., 1999) and acute ammonia challenge (Hawkins et al., 1973). It has also been demonstrated that retina produces more lactate per

volume of tissue than the cerebral cortex, which could relate to the relatively higher MCT4 expression in the former structure (Vina et al. 1997; Wang and Bill, 1997; Wang et al. 1997). Because of the deleterious effects resulting from accumulation of lactate and acidosis, it is important that lactate and protons are cleared rapidly. MCT4 localized in the glial endfeet is likely to play an important role in this process.

Glial endfeet are essential for the homeostasis of the brain. The endfeet are strategically positioned to regulate exchange of water, ions, and metabolites between brain and extracerebral liquid compartments (blood and cerebrospinal fluid) and cover >90 % of the capillary surface. They have a unique repertoire of transporters and ion channels, such as the water channel AQP4 and Kir4.1 (Nagelhus et al., 1999). Knockdown of Kir4.1 leads to a loss of K^+ homeostasis in the retina by interfering with K^+ spatial buffering (Kofuji et al., 2000). Also, removal of perivascular AQP4 affects water influx and reduces the size of infarction following MCA occlusion (Amiry-Moghaddam et al., 2003b). This emphasizes the important role of the glial endfeet for normal brain function and brain injuries.

It is currently unknown to what extent the glial endfeet are able to perform their functions following ischemia to maintain brain homeostasis. The astrocyte endfeet are anchored to the basal lamina by an integrin (heterodimer $\alpha_6\beta_4$) that has been shown to be significantly reduced during and following MCA occlusion (Wagner et al., 1997). This indicates that the integrins are sensitive to ischemia and that astrocyte endfeet may loose their attachment within hours of reperfusion. There are also observations that show that the endfeet swell following a transient ischemic episode (Ito et al., 1997). These studies suggest that important functions of endfeet may be impaired.

MCT1 was demonstrated by us and others to be expressed in the endothelial cells (Kido et al., 2000; Bergersen et al., 2002; Mac and Nalecz, 2003). This monocarboxylate transporter isoform is likely to act in concert with MCT4 to release lactate from the brain. MCT1 has different functional characteristics compared to that of MCT4, with higher affinity for lactate and other monocarboxylates. This difference in affinities may be important to create a gradient of lactate that drives lactate out into the blood. The expression of MCT1 has been demonstrated to be

increased following transient global ischemia and focal cerebral ischemia in the spontaneous hypertensive rat, suggesting that MCT1 may also be important for the removal of lactate and enhanced survival of the neurons in the post-ischemic brain (Tseng et al., 2003; Zhang et al., 2005).

Interestingly, the endfoot pool of MCT4 faces pericytes as well as endothelium. Pericytes show a high expression of contractile proteins and can provide potentially potent, decentralized sites at which capillary perfusion can be regulated to meet the brain's metabolic demands (Tilton et al., 1979; Schonfelder et al., 1998). MCT4 abutting on pericytes could mediate lactate as a signal that couple retinal/ brain activity and local cerebral blood flow. In favour of this hypothesis, an *in vitro* study has shown that lactate induced pericyte contraction in normoxia and relaxation in hypoxic conditions which could be blocked by monocarboxylate inhibitors (Yamanishi et al., 2006). Additional support comes from observations from Mintun *et al.* that demonstrated that blood flow in photo-stimulated human visual cortex is augmented by lactate injection (Mintun et al., 2004). This again suggest that the glial endfeet, possibly in close interaction with pericytes, are important mediators for fine regulation of local cerebral blood flow (Takano et al., 2006).

Our studies has demonstrated that many astrocytes are viable in the neocortical areas several hours after focal cerebral ischemia, thus appearing as very promising targets for future pharmacological interventions. The astrocyte endfeet are particularly interesting in this regard, given their strategical position at the brain-blood interface and their known roles in maintaining homeostatic functions and BBB integrity We will now investigate the expression of MCT4 following focal cerebral ischemia. Thus, we hypothesize that alterations in the expression of this protein may affect the clearance of lactate in the post-ischemic phase. In parallel studies we will explore the possibility that astrocytes up-regulate MCT4 in response to hypoxia, as part of a compensatory mechanism.

CONCLUSIONS

I. The recovery of the astrocytic metabolism of ^{14}C-acetate differed substantially between regions during the first four hours of reperfusion following temporary MCA occlusion. The pattern of recovery was similar after either 2 or 3 h of occlusion. The ^{14}C-acetate metabolism was substantially reduced in the striatal tissue. This is an area that forms part of the focal tissue and is subjected to severe ischemia during the occlusion period. In contrast, ^{14}C-acetate metabolism was essentially unchanged in perifocal tissue in the cerebral cortex, a region that had been subjected to moderate ischemia. A more variable response was seen in focal cortical tissue although the metabolism was largely preserved in the majority of rats. These results indicate that most astrocytes in the cortex remain viable at least for the first few hours after transient ischemia. Furthermore, the surviving astrocytes recover complex metabolic functions even in those areas that eventually will be incorporated in the infarction.

II. The production of ^{14}C-glutamate and ^{14}C-glutamine generated from [U-^{14}C]glucose metabolism was greatly reduced at 1 h of reperfusion following both 2 or 3 h of ischemia. All parts of the MCA perfusion territory were affected, and in some instances even, beyond this region. The decrease in ^{14}C-glutamate generation points to marked alterations in metabolic activity in neurons, probably arising from both a depression in glucose use and impairment of pyruvate oxidation. The decreased production of ^{14}C-glutamine could result in part from reduced glucose metabolism in astrocytes. However, it is likely that this decrease largely reflects a reduced delivery of ^{14}C-glutamate from neurons to the astrocytes.

III. The decreases in oxidative metabolism of ^{14}C-glucose during early reperfusion were not closely related to either the severity of ischemia to which the tissue was exposed or the likelihood that the tissue would subsequently become infarcted. PCr and ATP:ADP were largely restored throughout the MCA perfusion territory suggesting that energy needs were mostly being met despite the alterations in glucose metabolism. Tissue lactate content was increased. This change was greater following the longer ischemic period and was restricted to tissue regions that were destined to become infarcted.

IV. The lactate preferring transporter MCT4 was found to be enriched in glial perivascular endfeet in the CNS. High expression levels were observed in the retina, intermediate levels in the neocortex, and relatively low levels in the cerebellum. The characteristics and distribution of this transporter suggest an important role in the clearance of excess lactate from brain and retina.

FUTURE STUDIES

MCT4 expression following focal cerebral ischemia.

The expression of MCT4 will be examined in focal and perifocal areas exposed MCA occlusion (90 min) in mice. Several reperfusion time points will be studied (2, 6 and 12 h) using light microscopy and electron microscopy. By this approach we will identify changes in the quantity and distribution of the expression of MCT4 as the tissue progresses to become infarcted. Such information would also reveal if altered MCT4 expression contributes to the observed lactate accumulations in the post-ischemic tissue.

MCT4 expression during and following hypoxia exposure.

MCT4 expression will be studied in brains from mice exposed to 6 or 24 h of hypoxia to test whether MCT4 expression is up-regulated in response to increased lactate levels. The hypoxic exposure does not result in any permanent damage to the brain but increases the glycolytic activity as it becomes more dependent on anaerobic metabolism to meet its energy demands. The quantity of hypoxia inducible factor 1-α (HIF1- α) will also be investigated as it has been linked to MCT4 mRNA expression. In addition, the effect of reoxygenation following hypoxia will be studied as this could indicate if MCT4 is one protein that induces a preconditioning effect in tissues.

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