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Induction of Monocarboxylate Transporter 2 Expression and Ketone Transport following Traumatic Brain Injury in Juvenile and Adult Rats

M.L. Prins[a, c] C.C. Giza[a, b, c]

Divisions of [a]Neurosurgery and [b]Pediatric Neurology, and [c]Brain Injury Research Center, David Geffen School of Medicine at UCLA, Los Angeles, Calif., USA

Key Words
Monocarboxylate transporter 2 · Ketones · Traumatic brain injury

Abstract
Based on recent work demonstrating age-dependent ketogenic neuroprotection after traumatic brain injury (TBI), it was hypothesized that the neuroprotection among early post-weaned animals was related to induced cerebral transport of ketones after injury. Regional changes in monocarboxylate transporter 2 (MCT2) were acutely examined with immunohistochemistry after sham surgery or controlled cortical impact injury among postnatal day 35 and adult rats. Both ages showed elevated MCT2 expression in the ipsilateral cerebral vasculature after TBI. Using Western blotting, MCT2 expression was 80–88% greater in microvessels isolated from postnatal day 35 rats at all time points relative to adults. The increased MCT2 expression was temporally correlated with an age-related increase in cerebral uptake of ketones, when ketones were made available after injury.

Introduction

Ketone bodies have been shown to provide effective neuroprotection from various neuropathological conditions, including Parkinson's and Alzheimer's disease [1], hypoxia-ischemia [2–5], hemorrhagic shock [6] and glycolytic inhibition [7, 8]. More recently, our laboratory has demonstrated age-dependent neuroprotection among animals on a ketogenic (KG) diet following traumatic brain injury (TBI). In this study, younger post-weaned animals showed a greater decrease in cortical contusion volumes than adults with KG treatment or suckling animals [9]. This apparent beneficial role for ketones under neuropathological conditions implicates changes in cerebral ketone uptake.

Studies that increase ketone availability through dietary modulation or starvation have suggested that ketone transport across the blood-brain barrier (BBB) is the rate-limiting step in cerebral ketone utilization [10–13]. The monocarboxylate transporters (MCTs) are responsible for the transport of ketones, pyruvate and lactate across the BBB and cell membranes. Changes in the cerebral density of MCT1 and MCT2 have been reported acutely following hypoxia-ischemia in vivo and in hippocampal slices [14, 15], stroke [16], noradrenaline application in cultured neuronal cells [17] and substrate induction in colonic and brain tissue [18, 19]. In addition to induction by pathological conditions, alterations in MCT expression/function occur during normal cerebral development. While the natural cerebral capacity of the suckling animal to metabolize ketones decreases sharply after weaning, there is evidence for ongoing post-weaning developmental differences in cerebral ketone uptake [20], density of ketone transporters [21], transporter kinetics [22] and inducibility of transporter expression [13]. These findings suggest a greater capacity for younger post-weaned age groups to

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Mayumi L. Prins, PhD
Division of Neurosurgery, UCLA School of Medicine
Room NPI 18–228, Box 957039
Los Angeles, CA 90095-7039 (USA)
Tel. +1 310 825 0646, Fax +1 310 794 2147, E-Mail mprins@mednet.ucla.edu

rely on cerebral ketone metabolism when the substrate is available. Based on the collective evidence from development and neuropathology, we propose that TBI will induce changes in MCT2 expression and that these changes will be age dependent.

Materials and Methods

Subjects/Experimental Groups

Postnatal day (PND) 35 (121.5 ± 2.8 g) and adult (289.7 ± 2.4 g) male Sprague-Dawley rats were given either sham surgery or a lateral controlled cortical impact (CCI) injury. Regional changes in MCT2 expression were determined by immunohistochemistry for PND 35 and adult rats after sham surgery (n = 8 each), 6 h after CCI (n = 5 and 6, respectively) and 24 h after CCI injury (n = 6 each). Quantitative differences in MCT2 expression were determined by Western blot analysis of the microvessel fraction of the brain in PND 35 and adult rats after sham surgery (n = 5 each), 6 h after CCI (n = 5 each) and 24 h after CCI injury (n = 5 each).

In order to determine whether changes in MCT2 expression were associated with changes in cerebral uptake of ketones, a separate group of animals was placed on a KG diet immediately after injury, and arteriovenous samples were obtained at 24 h after sham surgery or CCI injury (PND 35 and adult sham standard fed, n = 7 and 5; sham KG diet, n = 5 and 6; CCI standard fed, n = 6 and 5; CCI KG n = 8 and 8, respectively).

CCI Injury

As characterized previously [23], the CCI injury model was used to generate a focal TBI to the left hemisphere. Under isoflurane (1.5–2.0%/100% O_2), a midline incision was made and the skull was exposed. A 6-mm-diameter craniotomy was drilled over the left hemisphere under a microscope, centered at –4 mm anterior-posterior and 5 mm lateral, relative to the bregma. The bone flap was removed and the dura left intact in all animals. An electronically controlled pneumatic piston cylinder (Hydraulics Control, Inc., Emeryville, Calif., USA) was mounted onto a stereotaxic micromanipulator (Kopf Instruments, Tujunga, Calif., USA) to allow for precise localization of the impact center. The piston cylinder was angled 22° away from vertical to allow the flat (5-mm-diameter) impactor surface to make contact perpendicular to the surface of the brain. The flat impactor tip compressed the brain 2 mm below the pial surface at 1.9 m/s. Following the injury, a small piece of gelfoam was placed over the craniotomy site to reduce bleeding.

Immunohistochemistry

MCT2 immunohistochemistry was performed to evaluate the regional changes in transporter expression at 6 and 24 h after TBI. Rats were perfused with 0.1 M phosphate-buffered saline (PBS)/4% paraformaldehyde, the brains removed and placed in 20% sucrose. Coronal frozen sections (40 μM) were collected and washed in PBS before preincubation in normal serum block (0.1% BSA/1.5% normal goat serum) for 1 h. Sections were incubated in the primary antibody (MCT2, Chemicon International, Temecula, Calif., USA, 1:1,000 dilution; SMI-71, Sternberger Monoclonals Inc., 1:1,000) for 1.5 h at room temperature followed by three PBS washes. Endogenous peroxidase activity was blocked by incubation in 0.5% H_2O_2 for 30 min followed by three PBS washes. Sections were then incubated in the secondary antibody (biotinylated anti-chicken immunoglobulin G in goat; Vector Laboratories, Burlingame, Calif., USA) for 30 min at room temperature followed by an avidin-biotin complex to which the horseradish peroxidase enzyme is bound, for an additional 30 min. The MCT2 and SMI-71 stains were developed using diaminobenzidine and vasoactive intestinal polypeptide substrate kits, respectively (Vector Laboratories). The sections were then mounted onto gelatin-coated slides, dried, dehydrated, coverslipped and analyzed microscopically. Minus primary controls and MCT2 peptide preabsorption (20 μg/ml; Chemicon International) were conducted in both PND 35 and adult tissue as controls for nonspecific binding and antibody specificity.

Microvessel Preparation

Under light gas anesthesia, animals were decapitated and trunk blood was collected and the brain dissected on ice. Ipsilateral cortical tissue was dissected, flash frozen and stored at –70°C. The ipsilateral cerebral cortices from 5 rats in a given group were pooled and processed for microvessel isolation as previously described [21]. The cortices were homogenized with a Teflon and glass homogenizer in microvessel buffer (MVB; 15 mM HEPES, 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, 0.5% BSA, pH 7.4) with Complete protease inhibitor cocktail (Roche Diagnotics, Mannheim, Germany). The homogenate was centrifuged at 2,000 *g* for 20 min at 4°C. The pellet was resuspended in MVB, layered on top of a 17% dextran solution (MW 38,000) and centrifuged at 5,400 *g* for 20 min at 4°C. The final pellet was poured over a nylon mesh screen (41 μm) and washed with a stream of cold MVB. The microvessel fraction was collected from the top of the screen and stored at –80°C.

Western Blotting

The samples were homogenized by sonic dismembrator in lysis buffer (62.5 mM Tris HCl, 50 mM dithiothreitol, 0.3% SDS, 1 mM PMSF, pH 7.4) containing Complete protease inhibitor cocktail (Roche Diagnotics). Protein concentrations were determined using DC protein assay (BioRad, Hercules, Calif., USA), and 2 μg were incubated for 5 min at 95°C in SDS-PAGE sample buffer (62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue) and loaded onto a 10% polyacrylamide gradient gel (BioRad). After electrophoresis, samples were transferred to nitrocellulose membranes (ImmunoBlot PVDF; BioRad), total protein signal on the blot was visualized using Sypro Ruby Red stain (BioRad) and digitally captured using a Fluor-S Max MultiImager (BioRad). The membranes were then blocked in TBS-T (0.1% Tween, 10% milk, 1% BSA in TBS) for 1 h at room temperature. Samples were incubated 18 h with the primary antibody to MCT2 (Chemicon International) diluted to 1:5,000 in TBS-T. The membranes were washed repeatedly with TBS-T for 1 h and then incubated with goat anti-chicken immunoglobulin Y conjugated to alkaline phosphate (Abcam Inc., Cambridge, Mass., USA) and diluted 1:250,000 for 1 h at room temperature. After repeated washes in TBS-T, bound antibody was visualized by chemiluminescence (ECL plus, Amersham, Piscataway, N.J., USA) on a Fluor-S Max MultiImager (BioRad). The MCT2 signals for each sample were normalized to total protein transferred to the blot in its respective lane and then reported as a percentage of the adult sham average.

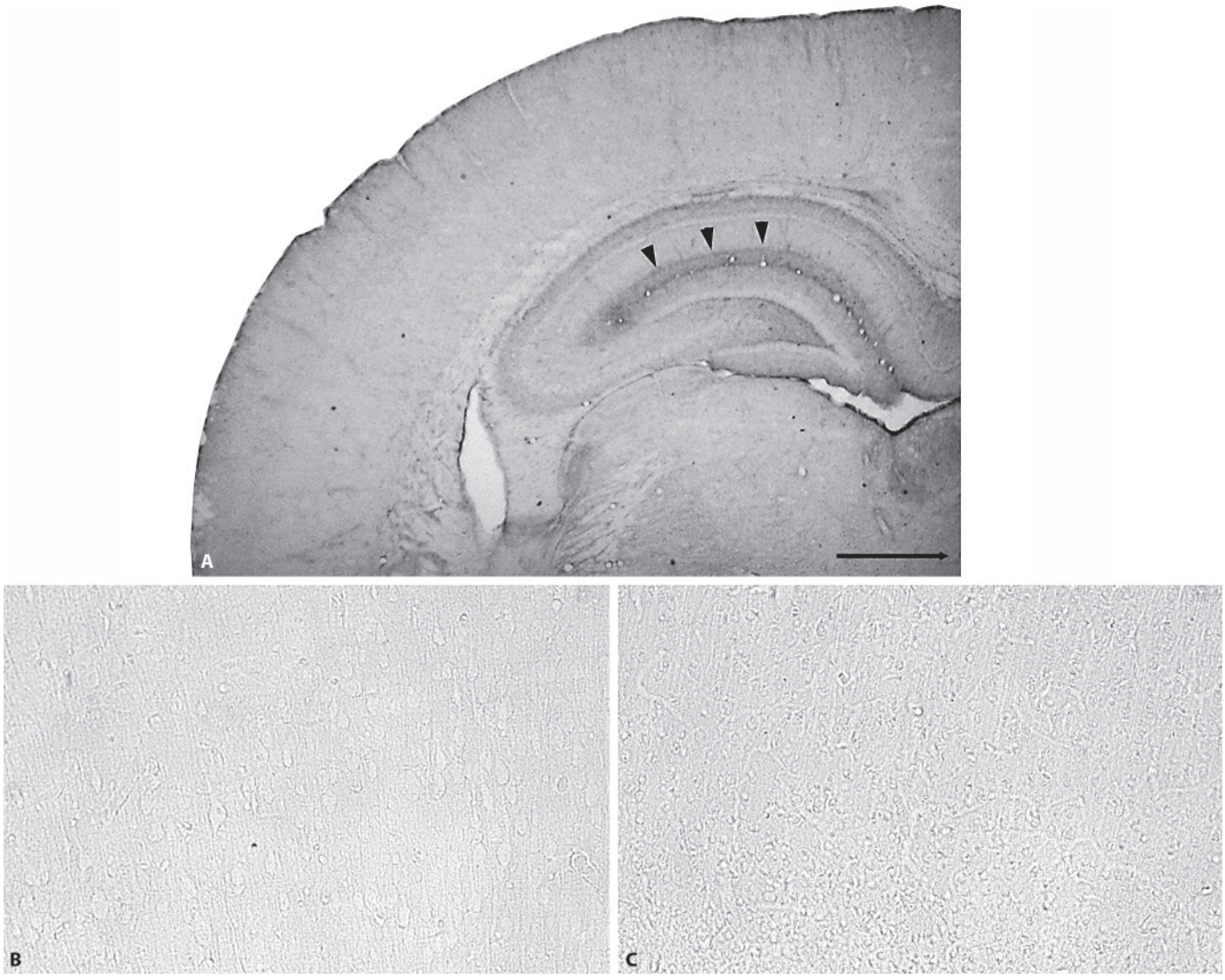

Fig. 1. MCT2 immunoreactivity in the coronal section of the adult rat brain (**A**). Lack of staining in minus primary control (**B**) and MCT2 peptide preabsorbed control (**C**). Diffuse staining is present throughout the cortex and hippocampus with intense labeling in the lacunosum moleculare of the hippocampus (arrowheads, **A**), consistent with previous studies.

Diets

Animals were given free access to water and either a standard diet of rodent chow (Teklad No. 7013) or the high-fat KG diet (Bioserv, Frenchtown, N.J., USA; No. F3666). Comparison of the constituents of both diets have been previously published [9]. The KG diet has been used to elevate blood concentrations of ketone bodies – β-hydroxybutyrate (βOHB) and acetoacetate – in normal rats [24] and following TBI [9].

Arteriovenous Sampling

Twenty-four hours after sham surgery or CCI injury, animals were placed under anesthesia to catheterize both the femoral artery and sagittal sinus for arteriovenous sampling. The arterial cannulation was performed with polyethylene tubing (PE50). The animal was then positioned into a stereotaxic frame, the skull surface was exposed and a 2-mm-diameter craniotomy was made 4 mm anterior to lambda on the midline. The bone flap was removed to expose the sagittal sinus. Under a surgical microscope, a 30-gauge needle tip was used to puncture the sinus and a chronic catheter (0.3 mm ID × 0.6 mm OD; Harvard Apparatus, Holliston, Mass., USA) was inserted towards the confluence of the sinuses. A piece of gelfoam was used to cover the craniotomy, and dental cement was applied to secure the cannulae. Blood samples (0.2 cm^3) were obtained from both lines. A 25-μl aliquot of whole blood from the artery and sinus were processed immediately for glucose and lactate concentrations on the YSI analyzer (YSI Inc., Yellow Springs, Ohio, USA). The remaining blood was centrifuged (12,000 *g* for 5 min) to obtain the plasma, which was stored frozen until processing for βOHB on the

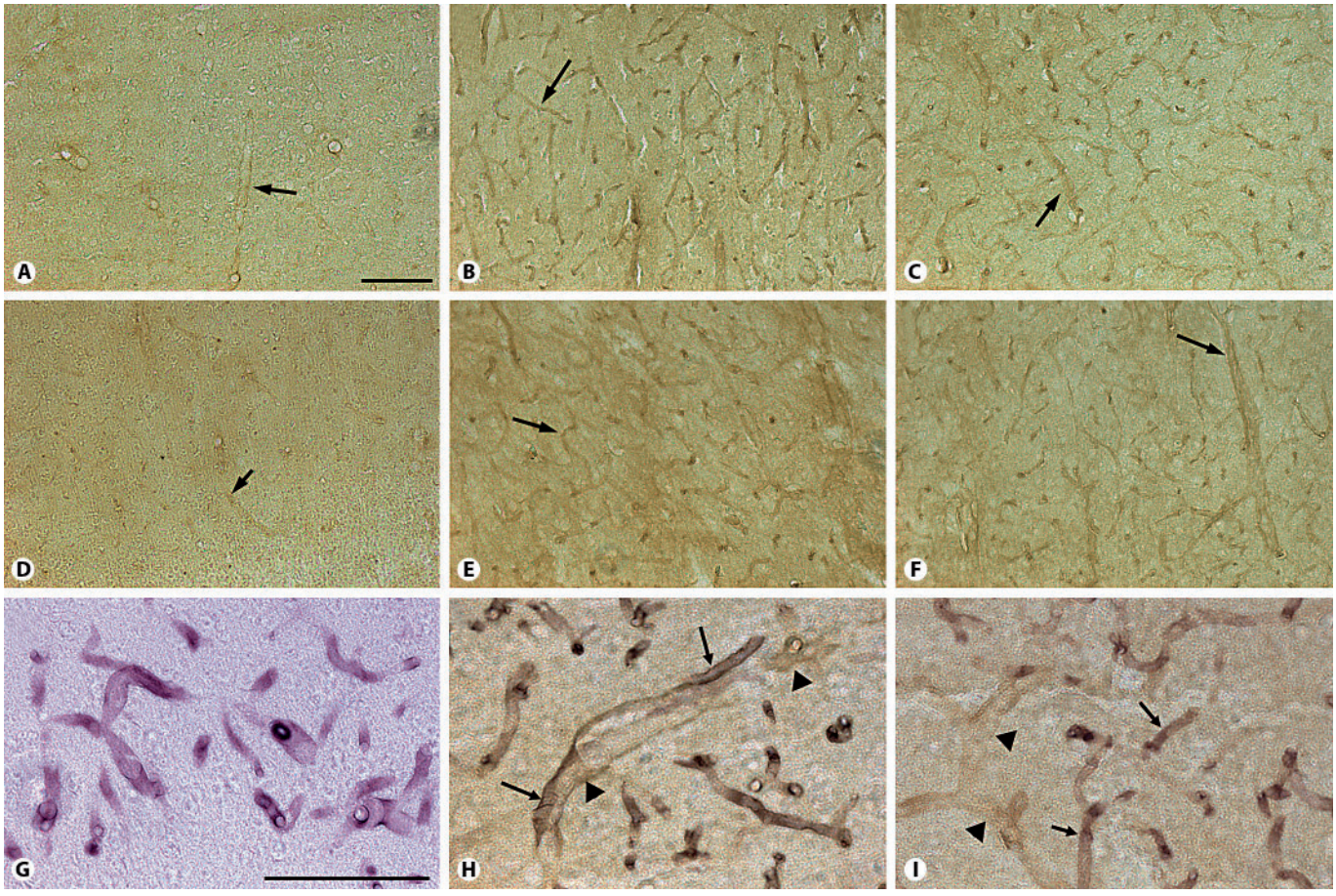

Fig. 2. Ipsilateral cortex labeled with MCT2 in sham, 6 and 24 h after CCI-injured PND 35 (**A–C**) and adult (**D–F**) rats, respectively. Arrows point to examples of labeled microvessels. **A–F** Objective ×10; scale bar = 0.1 mm. Ipsilateral cortical tissue from injured PND 35 rats showing vascular staining with SMI-71 (**G**) and SMI-71/MCT2 double labeling (**H**, **I**). Arrowheads show brown diaminobenzidine reaction of MCT2. Arrows show regions of overlap of the purple (SMI-71) and brown (MCT2) stain on the same vessels. **G–I** Objective ×20; scale bar = 0.1 mm.

GM7-Microstat analyzer (Analox Instruments, Lunenburg, Mass., USA). Each blood and plasma sample was run in triplicate.

Statistical Analysis

All data were expressed as mean ± SEM. Differences between groups were tested using one-way ANOVA, with Tukey's test for comparison between groups. Differences were considered statistically significant when $p < 0.05$.

Results

Immunohistochemistry

Standard-fed noninjured rat brain sections displayed a pattern of MCT2 immunoreactivity similar to previous reports (fig. 1A) [25]. The normal brain showed light diffuse MCT2 staining with small and large blood vessels positively labeled in the cortex and hippocampus. More intense immunoreactivity was observed in the lacunosum molecular layer of the hippocampus. Both the minus primary and preabsorbed peptide control sections showed no MCT2 staining (fig. 1B, C).

Sham animals in both age groups showed diffuse labeling with few positively labeled microvessels (fig. 2A, D). Following TBI, robust MCT2 immunoreactivity became apparent in microvasculature within the ipsilateral cortex (fig. 2B, C, E, F), hippocampus (fig. 3) and underlying thalamus. The TBI-induced MCT2 staining appeared to be primarily vascular, as shown at greater magnification using double labeling with the BBB marker SMI-71 (fig. 2G–I). Both the purple SMI-71 and the brown MCT2 label are

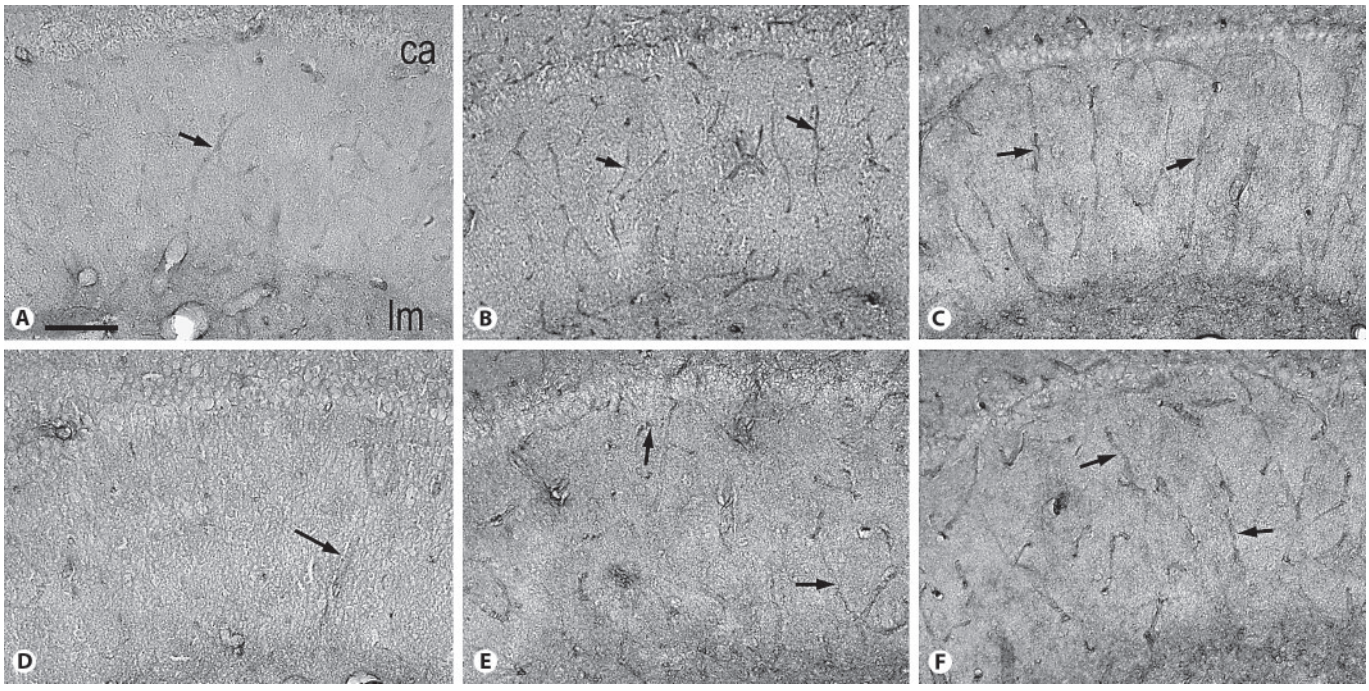

Fig. 3. Coronal sections showing the ipsilateral hippocampus labeled with MCT2 in sham, 6 and 24 h after CCI-injured PND 35 (**A–C**) and adult (**D–F**) rats, respectively. Arrows point to examples of labeled microvessels. ca = Pyramidal cell layer; lm = lacunosum moleculare. Objective × 10; scale bar = 0.1 mm.

seen colocalized (black) on the same vessels. This increase in MCT2 labeling was apparent at 3 h (data not shown) and 6 h (fig. 2B, E) after CCI injury in the cortex. While cortical sections continued to show robust staining at 24 h (fig. 2C, F), the ipsilateral hippocampus showed less labeling than after 6 h in PND 35 animals (fig. 3B, C).

The contralateral cortex, hippocampus and thalamus of some CCI-injured animals showed sham-like MCT2 labeling, while others showed more microvascular labeling. Despite the presence of some contralateral MCT2 immunolabeling, the ipsilateral staining was always significantly greater in both intensity and distribution. This finding was consistent in both age groups.

Western Blot Analysis

Age-related differences in MCT2 immunoreactivity were difficult to quantify with the vascular staining pattern. For this reason, the pooled ipsilateral cortical microvessel fraction was obtained for each experimental group, and MCT2 Western blot analysis was used to determine semiquantitative differences. PND 35 sham animals showed a 85% greater MCT2 expression than sham adults (fig. 4). Both age groups showed 26–28% increase in MCT2 expression in the microvessel fraction at 6 h after injury and 16% increase at 24 h relative to their age-matched shams.

Arterial and Arteriovenous Difference Changes

Arterial blood samples from standard-fed sham and injured animals showed no significant difference in plasma βOHB concentrations between juvenile and adult groups (fig. 5). The mean plasma βOHB level among standard-fed adults and PND 35 rats was 0.12 ± 0.01 and 0.13 ± 0.02 mM, respectively.

Arterial samples from animals fed with the KG diet for 24 h showed significant increase in plasma βOHB concentrations in both age groups relative to standard-fed animals (fig. 5). There was a significant age effect among sham KG-fed animals, with PND 35 achieving 1.56 ± 0.08 mM and adults reaching 0.74 ± 0.17 mM ($p < 0.01$) plasma ketone body concentrations. While an age effect was observed in the sham KG-fed group, there was no statistical difference in plasma βOHB attained at 6 ($p = 0.06$) or 24 h after injury between age groups.

Changes in cerebral uptake of βOHB, glucose and lactate among standard and KG-fed sham and injured ani-

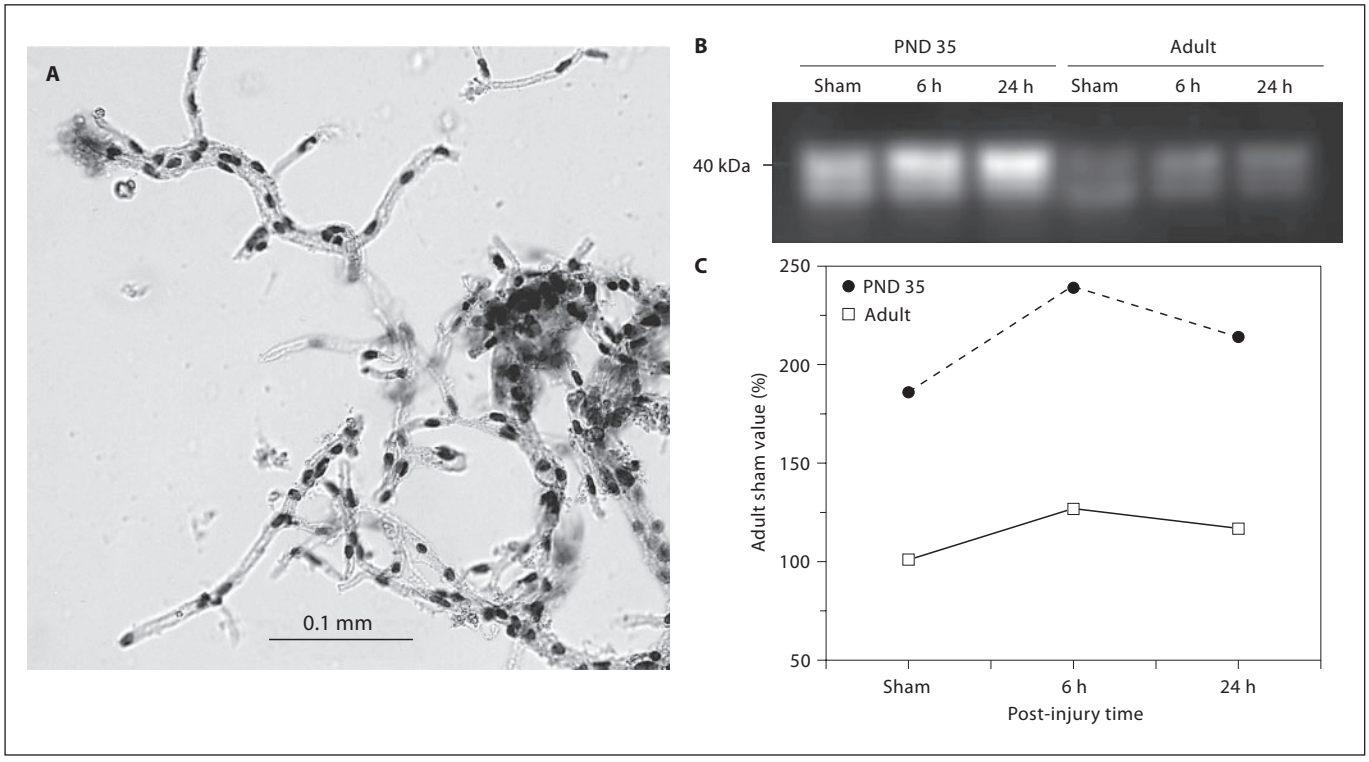

Fig. 4. MCT2 Western blot analysis on pooled isolated microvessel fractions of the ipsilateral cortex. Isolated microvessels stained with toluidine blue (**A**). Representative Western blot of MCT2 from sham, 6 and 24 h after CCI-injured PND 35 and adult brains (**B**). Western blot MCT2 signals (normalized to total protein in the same lane) were analyzed relative to adult sham animals (= 100%) and show the changes after injury in both age groups (**C**).

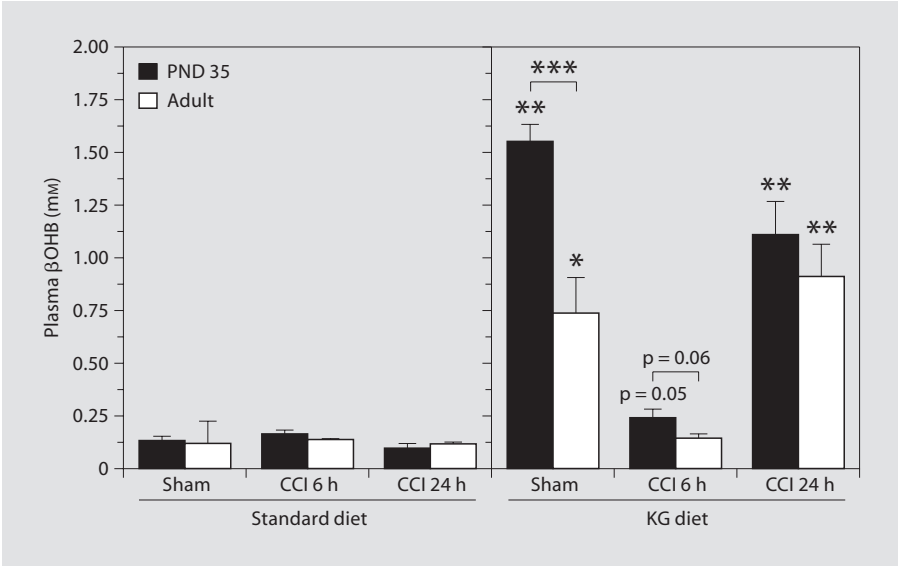

Fig. 5. Arterial plasma concentrations of βOHB (mean ± SEM) among standard and KG-fed PND 35 and adult rats at 6 and 24 h following CCI injury. * $p < 0.05$, ** $p < 0.01$ relative to age-matched standard-fed animals. *** $p < 0.01$ relative to adult KG fed sham animals.

Table 1. Cerebral arteriovenous difference of βOHB, glucose and lactate in adult and PND 35 rats at 24 h following CCI or sham injury (mean ± SEM)

	Sham standard diet		Sham KG diet		CCI injury standard diet		CCI injury KG diet	
	adult	PND 35	adult	PND 35	adult	PND 35	adult	PND 35
βOHB	0.03 ± 0.00	0.02 ± 0.01	0.05 ± 0.01	0.05 ± 0.03	0.04 ± 0.01	0.02 ± 0.01	0.07 ± 0.03	0.12 ± 0.02
		p = 0.06				*	**	
Glucose	0.70 ± 0.07	0.58 ± 0.08	0.47 ± 0.08	0.56 ± 0.15	0.68 ± 0.16	0.55 ± 0.10	0.35 ± 0.10	0.52 ± 0.05
							*	
Lactate	−0.04 ± 0.03	−0.07 ± 0.03	−0.07 ± 0.01	−0.13 ± 0.02	−0.08 ± 0.03	−0.08 ± 0.03	−0.15 ± 0.04	−0.16 ± 0.04

Positive arteriovenous difference indicates uptake and negative arteriovenous difference indicates release.
* $p < 0.05$; ** $p < 0.01$.

mals are shown in table 1. Adult and PND 35 sham animals on the KG diet for 24 h showed no significant change in cerebral uptake of βOHB, glucose or lactate compared with those on the standard diet. There was a trend for lower glucose in sham adults on the KG diet ($p = 0.06$). Adult animals placed on the KG diet for 24 h after CCI injury showed an increase in βOHB uptake compared with injured animals on a standard diet, but this increase did not reach significance. Unlike the adults, PND 35 injured animals on the KG diet showed a significant increase in βOHB uptake and significant release of lactate from the brain. While the injured adult KG-fed animals showed decreased glucose uptake, this was not observed among PND 35 KG-fed animals.

Discussion

The results of the current study demonstrate induction of MCT2 acutely after TBI, primarily within the cerebral microvasculature among both PND 35 and adult animals. The overall magnitude of the MCT2 expression was 85% greater in PND 35 animals relative to adults. In the presence of increased circulating ketones (a monocarboxylate), the increased transporter expression was temporally correlated with an age-related increase in cerebral uptake of ketones.

MCT2 Labeling of Cerebral Microvasculature

Cerebral localization of MCTs have previously been described in both the developing and adult brain [26–30]. In general, MCT1 was predominantly expressed in endothelia and astrocytes [29, 31, 32], whereas MCT2 demonstrated more neuronal localization [27, 33, 34]. It is important to note that while the general primary localization of each transporter has been described, they are not exclusive. Examination of reported immunohistochemical results demonstrated positive MCT2 immunoreactivity within cerebral microvessels [25, 27, 34–37]. Therefore, MCT2 labeling in cerebral endothelia in the current study is consistent with previous findings.

Dynamic Changes in MCTs

The brain has the ability to regulate the expression of MCTs under both physiological (development, changes in substrate availability) and pathological conditions (hypoxia, ischemia, TBI). Changes in the expression of both MCT1 and MCT2 have been reported in numerous tissue types, including brain, and have been induced by diet, substrate presence, ischemia, starvation or chemical stimulation (table 2) [15–19, 38–42]. While several studies reported changes after 4–6 weeks, the majority demonstrated changes in MCT expression within hours and days. The current study is the first to document acute changes in MCT2 expression following TBI and, in conjunction with previous studies, demonstrates the potential for dynamic changes in MCT expression. The ability for rapid induction of MCTs may be attributed to its reported regulation at the translational level rather than requiring transcription, as demonstrated in neuronal cultures by Pierre et al. [17].

TBI-Induced MCT2 Expression

While studies have demonstrated the cerebral capacity to alter MCT protein expression, the mechanisms regulat-

Table 2. Literature demonstrating dynamic changes in MCT expression induced by diet, injury or chemical stimulation in various tissue types

Tissue/cell type	Inducing factor	Change	Time	Reference
Brain endothelium	KG diet	8-fold increase in MCT1 protein	4 weeks	[19]
Cultured neurons	repeat hypoglycemia	increase in MCT protein is suggested by 4CIN blockade of adaptation to hypoglycemia	1 h	[39, 40]
Myocardial	myocardiac infarction	260% increase in MCT1 protein	6 weeks	[38]
Brain	ischemia	increase in MCT1 protein	3–24 h	[16]
Brain astroglia, endothelia	ischemia	increase in MCT1 protein	3–21 days	[15]
Testis	decrease in FSH	8-fold increase in MCT2 mRNA	2 days	[42]
Cortical neuronal culture	noradrenaline	250% increase in MCT2 protein, but not mRNA	6 h	[17]
Colonic epithelium culture	NaButyrate	5.7-fold increase in MCT1 mRNA 5.2-fold increase in MCT1 protein	12 h	[18]
Melanoma	low extracellular pH	increase in MCT1 protein, but not mRNA	1 h	[41]

FSH = Follicle-stimulating hormone; 4CIN = cyano-4-hydroxycinnamate.

ing these changes in the brain remain unclear at the present time. Studies involving synaptic activity-induced MCT2 upregulation showed increased protein levels with noradrenalin, cAMP and forskolin application in cortical neurons [17]. These data suggest a cAMP/protein kinase A mediated pathway in regulating MCT2 expression. Interestingly, this pathway has recently been shown to play a role in decreasing the rate of lactate transport through MCT1 in cerebral endothelial cells [43]. In addition to this pathway, activation of the hypoxia-inducible factor 1 (HIF1) binding site has been proposed to mediate MCT1 expression after hypoxia/ischemia [16, 44]. Both hypoxia and application of vascular endothelial growth factor increase MCT1 expression and are mediated by HIF1. It is still unclear at this time whether there is a common underlying mechanism for induction for both MCT1 and MCT2 or whether separate activation pathways exist. It is currently known that TBI increases vascular endothelial growth factor [45], HIF1 [46] and noradrenalin [47], and these factors may be involved in MCT2 induction.

Regardless of the mechanism for inducing MCT2 expression after TBI, the question remains whether a functional consequence exists to the increased transporter density. The arteriovenous measurements in the current study indicate that the elevated MCT2 expression coincides with increased uptake of ketones, when the substrate was made available, suggesting that the transporters have functional significance. This consequent change following TBI can be advantageous in delivery of alternative substrates to the brain following injury. However, future studies are needed to determine what proportion of the uptake is directly related to transport or diffusion, how TBI-induced BBB disruption may affect this uptake at different times after injury, and how KG diet alone affects MCT2 expression.

Age-Related Differences in Ketone Transport

Age-related differences in cerebral transport of ketones, lactate and glucose have been described following KG diet or starvation [10–12, 20]. However, the current study is the first documentation of age-related differences in ketone transport following TBI. The arteriovenous differences for ketones and the immunohistochemistry show greater substrate uptake and transporter expression in the younger animals. Despite achieving similar plasma levels of βOHB at 24 h, PND 35 animals showed a 6-fold increase in cerebral uptake of ketones after TBI compared with a 2-fold increase seen in adults. This increase in cerebral ketone uptake temporally coincides with increased MCT2 transporter expression; however, changes in MCT1 may also be contributory and are currently under study.

Age-dependent differences in brain uptake of substrates have been well described during development and dietary alterations. The results of the current study ex-

pand these age differences not only to the post-weaned age group, but also following TBI. We believe the concept of age-dependent differences in upregulation of MCT transporters may account for age differences in KG neuroprotection observed in our previous study and that these findings emphasize the therapeutic potential of ketones following TBI.

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