Diet-induced ketosis increases capillary density without altered blood flow in rat brain

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Puchowicz MA, Xu K, Sun X, Ivy A, Emancipator D, LaManna JC. Diet-induced ketosis increases capillary density without altered blood flow in rat brain. Am J Physiol Endocrinol Metab 292: E1607–E1615, 2007. First published February 6, 2007; doi:10.1152/ajpendo.00512.2006.— It is recognized that ketone bodies, such as β-hydroxybutyrate (β-HB) and acetoacetate, are energy sources for the brain. As with glucose metabolism, monocarboxylate uptake by the brain is dependent on the function and regulation of its own transporter system. We concurrently investigated ketone body influx, blood flow, and regulation of monocarboxylate transporter (MCT-1) and glucose transporter (GLUT-1) in diet-induced ketotic (KG) rat brain. Regional blood-to-brain β-HB influx (μmol·g⁻¹·min⁻¹) increased 40-fold with ketosis (4.8 ± 1.8 plasma-β-HB; mM) in all regions compared with the nonketotic groups (standard and no-fat diets); there were no changes in regional blood flow. Immunohistochemical staining revealed that GLUT-1 density (number/mm²) in the cortex was significantly elevated (40%) in the ketotic group compared with the standard and no-fat diet groups. MCT-1 was also markedly (3-fold) upregulated in the ketotic group compared with the standard diet group. In the standard diet group, 40% of the brain capillaries stained positive for MCT-1; this amount doubled with the ketogenic diet. Western blot analysis of isolated microvessels from ketotic rat brain showed an eightfold increase in GLUT-1 and a threefold increase in MCT-1 compared with the standard diet group. These data suggest that diet-induced ketosis results in increased vascular density at the blood-brain barrier without changes in blood flow. The increase in extraction fraction and capillary density with increased plasma ketone bodies indicates a significant flux of substrates available for brain energy metabolism.

ketone bodies; monocarboxylate transporter-1; GLUT-1; β-hydroxybutyrate; brain metabolism; blood-brain barrier

GLUCOSE IS THE MAJOR METABOLIC FUEL for the mammalian brain (37, 41), but alternate energy substrates such as ketone bodies, e.g., R-β-hydroxybutyrate (β-HB) and acetoacetate (6, 19, 29, 39), serve as efficient metabolic fuel sources for the brain. This is especially important under certain nutritional conditions, such as fasting, starvation (28), feeding of a high-fat diet (24, 26), and early development and throughout the suckling period (2, 25, 42). Clinicians and investigators have been interested in ketone bodies as therapeutic agents for the treatment of hypoglycemia, seizure disorders, and Alzheimer’s and Parkinson’s diseases and as alternatives to high-lipid parenteral and enteral feedings (5, 14, 43).

Similar in chemical structure to lactate and pyruvate (intermediates of glycolysis), ketones are metabolized by the brain, especially neonatal mammalian brain (1, 2, 20, 40). These short-chain acids are referred to as monocarboxylates, and, as with glucose metabolism, systemic delivery of these substrates and availability for neuronal uptake are highly dependent on their transport across the blood-brain barrier (BBB) through specific carrier-mediated facilitated transport processes. The first step to monocarboxylate entry at the BBB is through the monocarboxylate transporter (MCT-1) (3, 6, 42), which is the primary carrier-mediated transporter at the endothelial cell boundary of the BBB, analogous to the well-characterized transport system for D-glucose, GLUT-1 (7, 11, 31, 41).

Using the then-novel brain uptake index method, Oldendorf (27) was the first to describe the carrier-mediated BBB transport of short-chain monocarboxylic acids. Subsequent work in rats led to a modified uptake method, known as the single-pass dual-label isotope-indicator (unidirectional tracer) method, which allows for quantitation of substrates, such as glucose, lactate, or ketones, at the endothelial boundary of the BBB, as well as simultaneous estimation of blood flow (7, 16, 33).

MCT regulation in brain is dependent on nutritional status, especially during ketosis, such as that induced by diet (19), stage of development (6, 18, 42), and cell type (4, 8, 18, 23, 36, 42). MCT-1 and MCT-2 are known to play major functional roles in monocarboxylate metabolism in the brain in nonvascular and vascular regions. For example, MCT expression in rat brain has been associated with a role in the metabolic recovery following an ischemic insult (36, 45). However, the molecular and biochemical mechanisms underlying the regulation of MCT proteins in the brain, especially during a chronic state of ketosis, remain to be discerned.

It is unknown whether the influx of ketones across the BBB is related to changes in MCT-1 expression. The aim of this study was to assess MCT-1 transport capacity at the endothelial boundary of the BBB during chronic ketosis induced by diet. This study involved simultaneous measurement of the regional blood-to-brain ketone body uptake and blood flow across the BBB during elevated levels of blood ketones in a rat model of chronic ketosis. Using a single-pass dual-label isotope-indicator method, we measured blood-to-brain β-HB influx and blood flow in cortical, hippocampal, cerebellar, and brain stem regions. To determine whether changes in β-HB influx from blood to brain were a result of changes in blood flow and/or MCT protein expression, MCT-1 at the BBB was investigated under the condition of ketosis. The relation of MCT-1 to GLUT-1 was also studied by immunohistochemical analysis. This approach allowed for the concurrent assessment of changes in capillary density (10) and the coregulation of these two principal energy substrate transporters.

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MATERIALS AND METHODS

Animal preparation. All procedures were performed in strict accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. Male Wistar rats (28 days old, 90–130 g body wt) were allowed to acclimate in the Case Western Reserve University animal facility for 1 wk before they were used in experiments. Rats were then assigned randomly to three groups fed ad libitum for 4–6 wk as follows: the KG group was fed a ketogenic (high-fat, no-carbohydrate) diet, the CHO group was fed a high-carbohydrate, low-fat diet, and the STD group was fed standard laboratory chow (Table 1). The traditional (STD) diet (no. 8664, Teklad) was used as a control to the KG diet, and the CHO diet was used as an additional control to match micronutrient and protein components of the KG diet [diet nos. D12359 (CHO) and D12369b (KG), Research Diets, New Brunswick, NJ; info@ResearchDiets.com]. Before feeding was initiated, all the rats were fasted for 24 h to deplete glucose stores and initiate a state of ketosis.

Surgical procedures. On the day of the β-HB influx and blood flow experiments, nonfasted rats were anesthetized with 4% chloral hydrate (10 ml/kg ip; Fisher Scientific, Fair Lawn, NJ) during the surgical preparation and for the duration of the experiment, as previously described (17). Use of nonvolatile anesthesia enabled us to compare results with our previous measurements of regional blood flow in rat brain. Briefly, the surgical procedure involved placement of a right atrial silicone catheter (0.025 in. ID, 0.047 in. OD) into the external jugular vein and its insertion into the right atrium. A second polyethylene catheter (PE-50) was inserted into the femoral artery to monitor arterial blood pressure and obtain blood samples. Plasma samples from rats used for the β-HB influx and blood flow experiments were analyzed for glucose, lactate, and ketones (β-HB) by standard enzymatic assays on a Cobas Faras centrifuge analyzer-spectrophotometry system (Roche). In the rats used for immunohistochemical and immunocytochemical analyses, a small blood sample taken by tail stick from the KG group was used to determine the extraction fraction, i.e., ratio of tracer in tissue to tracer in arterial blood ([14C/3H]tissue/[14C/3H]art). Disintegrations per minute (dpm) in brain tissue samples were corrected (dpm_correct) for intravascular compartment volumes (Vpl) as follows: dpm_correct = dpm_tissue − (dpm_pl/μl × Vpl × tissue wt (g)) where Fpl is the specific activity of injected tracer, ρpl is the density of injected tracer, and Vpl is the intravascular volume. The fractionation correction was determined using immunohistochemistry stained for GLUT-1 and MCT-1. 

Regional blood-to-brain β-HB influx and blood flow calculations. Blood flow (BF; μl g−1 min−1) and β-HB influx (J; μmol·g−1 min−1) were determined from the measured radioisotope contents in each of the brain tissue and blood samples as follows (17)

\[
BF = F_p \times \left[ ^{3}H \right]_{tissue} / \left[ ^{3}H \right]_{tissue wt (g)}
\]

where Fp is the specific activity of injected tracer, ρpl is the density of injected tracer, and Vpl is the intravascular volume.

Immunohistochemistry. Since all capillaries highly stain for GLUT-1, indicative of the GLUT-1 transporter as a marker of the BBB changes in capillary density, rats were analyzed for cortical capillary density using immunohistochemical staining for GLUT-1 (STD, CHO, and KG groups, n = 4 per group), as previously described (10, 32). The protein expression of MCT-1 in capillaries at the BBB was also determined using immunohistochemistry. Briefly, rats were deeply anesthetized and transcardially perfused with PBS (pH 7.4) and then with 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde for 1 h, and embedded in paraffin. The coronal-cortical serial sections (5 μm, average of 3 sections, over 200 μm area per animal) were made from the full depth of the frontal cortex (beginning at bregma 1.6 mm, approximately plates 12–16). Paxinos-Watson stereotaxic coordinates, deparaffinized, rehydrated, and subjected to antigen retrieval in a microwave oven for 10 min using 0.01 M sodium citrate buffer (pH 6.0; Sigma). Endogenous peroxidase activity was blocked with 1% H2O2 in methanol for 10 min.

Sections were washed with PBS, incubated with 10% normal serum for 1 h, and incubated overnight at 4°C with a chicken anti-MCT-1 polyclonal antibody raised against the COOH-terminal 15 amino acids (1:300 dilution; Chemicon) or goat anti-GLUT-1 polyclonal antibody raised against a peptide mapping within the COOH terminus (1:200 dilution; Santa Cruz Biotechnology). Subsequently, they were incubated with biotinylated anti-chicken IgG or anti-goat IgG antibody (1:200 dilution; Vector Laboratories) and ABC complex solution.

Table 1. Diet compositions

<table>
<thead>
<tr>
<th></th>
<th>Fat</th>
<th>Protein</th>
<th>Carbohydrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG</td>
<td>89.5</td>
<td>10.4</td>
<td>0.1</td>
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<tr>
<td>CHO</td>
<td>11.5</td>
<td>10.4</td>
<td>78.1</td>
</tr>
<tr>
<td>STD</td>
<td>27.5</td>
<td>20.0</td>
<td>52.6</td>
</tr>
</tbody>
</table>

KG, ketogenic [high-fat (Crisco), no carbohydrate] diet; CHO, high-carbohydrate (dextrose), low-fat diet; STD, standard laboratory chow.

Table 2. Physiological parameters

<table>
<thead>
<tr>
<th></th>
<th>STD (n = 7)</th>
<th>CHO (n = 6)</th>
<th>KG (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma β-HB, mM</td>
<td>0.16 (0.2)</td>
<td>0.24 (0.2)</td>
<td>4.8 (1.8)</td>
</tr>
<tr>
<td>Plasma glucose, mM</td>
<td>11.5 (5.0)</td>
<td>10.0 (2.4)</td>
<td>11.3 (3.0)</td>
</tr>
<tr>
<td>mABP, mmHg</td>
<td>96.4 (4.7)</td>
<td>97.3 (5.9)</td>
<td>101.1 (6.7)</td>
</tr>
<tr>
<td>Pao₂, mmHg</td>
<td>87.1 (5.5)</td>
<td>86.2 (4.5)</td>
<td>87.5 (7.9)</td>
</tr>
<tr>
<td>Paco₂, mmHg</td>
<td>46.8 (4.4)</td>
<td>44.2 (1.0)</td>
<td>40.0 (4.2)*</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 (0.03)</td>
<td>7.3 (0.02)</td>
<td>7.3 (0.04)</td>
</tr>
<tr>
<td>Hct, %</td>
<td>47.4 (3.3)</td>
<td>48.2 (1.6)</td>
<td>47.5 (2.3)</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>168 (28)</td>
<td>163 (30)</td>
<td>153 (19)</td>
</tr>
</tbody>
</table>

Values are means (SD); n, number of rats. β-HB, β-hydroxybutyrate; mABP, mean arterial blood pressure; Pao₂, and Paco₂, arterial P O₂ and P CO₂. *Significantly different from STD and CHO.
(Vectastain ABC Kit, Vector Laboratories). Sections were developed in diaminobenzidine and H2O2 solution for color detection. Images of full-depth cortical sections were digitized with a SPOT digital camera connected to a Nikon E600 Eclipse microscope with x200 magnification. A computer image analysis system (Image Pro Plus, San Diego, CA) was used to detect MCT-1- and GLUT-1-positive staining, and microvessel (capillary) profiles were quantified by an experimentally blinded observer and expressed as number per square millimeter (15).

Colocalization of MCT-1 to GLUT-1 was assessed by double immunocytochemical labeling for MCT-1 and GLUT-1 to analyze immunoreactivities of the cortical sections. Sections were dewaxed, rehydrated, washed in PBS, and subjected to antigen retrieval. Slides were blocked with 10% normal donkey serum for 1 h at room temperature. Then the sections were treated with a mixture of the two primary antibodies, chicken anti-MCT-1 polyclonal antibody (1:300 dilution) and goat anti-GLUT-1 polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology), overnight at 4°C. The sections were washed and incubated with fluorescein-conjugated secondary antibody solution (Jackson ImmunoResearch Laboratories), which consisted of FITC-conjugated anti-chicken IgY (IgG) antibody (1:150 dilution) for MCT-1 (green) and Cy3-conjugated anti-goat IgG antibody (1:200 dilution) for GLUT-1 (red), for 1 h. Slides were rinsed and immediately mounted with Vectashield mounting medium (Vector Laboratories). Green and red light wavelengths were used to observe the stained sections, and the images were obtained with a SPOT digital camera connected to a Nikon E600 Eclipse microscope with x400 magnification.

Isolation of brain microvessels. Rat microvessels from the cerebral cortex (STD, CHO, and KG groups, n = 4) were isolated using a method previously described, with slight modifications (38). Briefly, after the animals were decapitated, the brains were quickly removed, and the cortical mantles were homogenized in 10 vol of ice-cold microvessel buffer (MB; 15 mM HEPES, 147 mM NaCl, 4 mM KCl, 3 mM CaCl2, 12 mM MgCl2, 5 mM glucose, and 0.5% BSA) supplemented with a protease inhibitor cocktail tablet (Roche Applied
Fig. 2. Regional ketone body extraction fraction in brain (14C-to-3H ratio in tissue vs. blood) in KG, STD, and CHO groups. Extraction fraction of β-HB was greatest in the KG group in all regions (cortical, hippocampal, cerebellar, and brain stem). Frontal and parietal extractions were significantly elevated in the KG group compared with the STD and CHO groups. Values are means (SD). *Significantly different from STD and CHO; ‡significantly different from cerebellar \((P < 0.05)\).

Fig. 3. Regional brain blood flow in KG, STD, and CHO groups. Blood flows were measured at β-HB plasma concentrations (mM) of 0.16 (SD 0.02), 0.24 (SD 0.2), and 4.8 (SD 1.8) in STD, CHO, and KG groups, respectively (see Table 2). Diet composition did not affect regional blood flows. Blood flows were lowest in the cerebellar region. Values are means (SD). *Significantly different from frontal and parietal; ‡significantly different from brain stem \((P < 0.05)\).
Fig. 4. Monocarboxylate transporter-1 (MCT-1) and GLUT-1 immunoreactivities in coronal-cortical sections in KG, STD, and CHO groups. A: MCT-1 and GLUT-1 immunohistochemical staining was visible in all groups. For MCT-1 and GLUT-1, intensity was greatest in the KG group. Increased intensity of GLUT-1 staining in the KG group indicates increased cortical capillary density. Images are representative of results from 4 observations. B: MCT-1 and GLUT-1 density analyses from coronal-cortical sections in A. Positive staining is expressed as number/mm². MCT-1 immunoreactivity was 3-fold greater in the KG group than in the STD and CHO groups. Capillary density (as measured by GLUT-1 staining) increased by 40% in the KG group compared with the STD and CHO groups. Ratio of MCT-1 to GLUT-1 increased (1:1) in the KG group. Values are means (SD). *P < 0.05 vs. STD and CHO.
and lactate concentrations were not significantly different among the diet groups.

The diet conditions did not affect the physiological parameters pH, P\textsubscript{aCO\textsubscript{2}}, arterial P\textsubscript{O\textsubscript{2}}, hematocrit, mean blood pressure, and body weight, except in the KG group, where P\textsubscript{aCO\textsubscript{2}} was slightly, but significantly, lower than in the other two diet groups (Table 2). This change in P\textsubscript{aCO\textsubscript{2}} was most likely not physiologically significant, since the arterial blood pH remained within normal limits. All rats in each of the groups gained weight consistently over the experimental period. Weight gain was less in the KG group than in the other two groups, but there were no significant differences among diet groups. The physiological data were similar to those we previously reported in chloral hydrate-anesthetized rats (16, 17, 35).

\(\beta\)-HB influx and extraction fraction. Regional blood-to-brain \(\beta\)-HB influx \(\left(\text{\textmu mol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}\right)\) was \(-40\)-fold greater in all regions in the KG group than in the nonketotic groups (Fig. 1A). In the KG group, the average cortical influx was 0.45 (SD 0.07) \((n = 13)\), whereas influx in the hippocampal, cerebellum, and brain stem regions \[0.38 \text{ (SD 0.10)}, 0.42 \text{ (SD 0.07)}, \text{ and 0.35 (SD 0.07)}, \text{ respectively}\] was slightly lower, but not significantly different from, that in the cortex. The regional variations in \(\beta\)-HB influx were greatly in the KG group than in the CHO and STD groups, reflective of the wide range of ketosis at plasma \(\beta\)-HB concentrations of 1.4–8.3 mM. Within each dietary group, there were no differences in regional \(\beta\)-HB influxes.

Since influx (see equation in MATERIALS AND METHODS) is a function of plasma \(\beta\)-HB concentration, we assessed the \(\beta\)-HB transport profile in the cortex of the KG group. Figure 1B shows a nonlinear, Michaelis-Menten-type relation of \(\beta\)-HB influx in the cortex as a function of plasma \(\beta\)-HB concentration; influx correlated \((R^2 = 0.8)\) with plasma \(\beta\)-HB concentration \((1.4–8.3 \text{ mM})\), at a ratio of \(\text{~}10:1.1\). Thus, for every 1.0 mM plasma \(\beta\)-HB, there was an influx of 0.1 \(\text{\textmu mol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}\) from blood to brain. This relation was similar in other regions (data not shown). The diffusional component was not determined in this study, inasmuch as it requires a nonstereospecific substrate, and little is known about the stereospecificity of MCT and ketones (e.g., \(S\)-\(\beta\)-HB) in the brain.

Regional brain extraction fractions in each of the diet groups are shown in Fig. 2. Extraction of \(\beta\)-HB from blood to brain at the BBB in all regions of the brain was greater in the KG group than in the nonketotic groups. There was a significant \((\sim50\%)\) increase in \(\beta\)-HB transport from blood to brain in the cortical regions (frontal and parietal) and an \(\sim30\%)\) increase in the hippocampal region in the KG group compared with the STD and CHO groups. There were no significant differences in regional extraction fractions between the STD and the CHO group. Comparison of the regional averages of all three diet groups showed a significantly lower extraction fraction in the brain stem than in the cerebellar region, but not in the other brain regions.

Regional brain blood flow. Regional brain blood flows \(\text{(mL} \cdot \text{g}^{-1} \cdot \text{min}^{-1})\) in each of the diet groups are shown in Fig. 3. Diet composition (STD, CHO, or KG) did not affect regional blood flows, inasmuch as there was no significant difference among the regions. The trends in regional blood flows in the STD group showed that blood flow was highest in the cortex \[1.1 \text{ (SD 0.1)}\] and brain stem \[1.4 \pm 0.2\] and lowest in the cerebellum \[0.90 \text{ (SD 0.2)}\]. Regional blood flows measured in this study were comparable to those previously reported using the blood flow-indicator method (16, 17, 35, 44).

\(MCT-1\) immunoreactivity and capillary density profiles. \(MCT-1\) and GLUT-1 immunohistochemical staining was visible in coronal-cortical sections from brains of rats fed the three diets (Fig. 4A). \(MCT-1\) immunoreactivity profiles and cerebral capillary density (identified by GLUT-1 immunostaining) were determined from the number of positive counts per unit area \((\text{number/mm}^2)\) (Fig. 4B). \(MCT-1\) staining appeared to predominate in the microvessels, as previously described (4, 6), with the greatest number of positive counts for \(MCT-1\) in the KG group (Fig. 4). \(MCT-1\) increased threefold in the KG group compared with the STD group: 428.7 \((\text{SD 36.8})\) vs. 138.4 \((\text{SD 10.4})\) (Fig. 4B). Capillary density (as shown by GLUT-1 staining) increased \(40\%\) in the KG group compared with the STD group (Fig. 4B). The capillary density profile in the STD group was similar to that previously reported (9, 32). Immunoreactivity was similar for GLUT-1 and \(MCT-1\) in the CHO and STD groups.

\(MCT-1\) and GLUT-1 expression in isolated microvessels. Changes in \(MCT-1\) and GLUT-1 protein content at the BBB were further assessed by Western blot analysis of isolated microvessels, which revealed a threefold increase in \(MCT-1\) and an eightfold increase in GLUT-1 in the KG group, compared to the STD group.
pared with the STD group (Fig. 5). Although expression was not as prevalent as in the KG group, the CHO group also showed an upward trend in these proteins in the isolated microvessels compared with the STD group that was not evident by immunoreactive staining.

**Colocalization of MCT-1 and GLUT-1.** Labeling of MCT-1 (green) and GLUT-1 (red) was observed in all diet groups, with the greatest immunoreactivities for MCT and GLUT in the KG group (Fig. 6). Since there were no observable differences between the CHO and the STD group, data for the CHO group are not shown. Colocalization of MCT-1 and GLUT-1 was increased in the KG group, as indicated by the yellow immunofluorescence, compared with the STD group. These data are consistent with the results in Fig. 5, which shows an increase in the number of capillaries, as well as in the ratio of MCT-1 to GLUT-1.

**DISCUSSION**

Chronic ketosis in rats induced by a KG diet resulted in a 20-fold increase in plasma $\beta$-HB. These levels were similar to those in humans fed a KG diet or fasted for 3 days. The increase in $\beta$-HB levels was associated with a 40-fold increase in $\beta$-HB brain influx and an increase in brain capillary density (measured by GLUT-1 immunostaining) without changes in regional blood flow or plasma glucose levels. Brain capillary MCT-1 also increased relative to the capillary density and content measured in the STD group.

Since ketone body influx into the brain is largely dependent on the blood concentration of ketones (12, 26, 29) and the activity of the MCT system (6), we expected that an increase in plasma $\beta$-HB concentration would result in increased $\beta$-HB influx across the BBB. A significant increase in plasma $\beta$-HB concentrations, as well as an increase in $\beta$-HB uptake in the brain, was observed. $\beta$-HB uptake in our adult ketotic rat model was consistent with brain ketone uptake with increased plasma ketone levels in humans (26, 29).

Similar to our findings, in an MR spectroscopy study, a 20-fold increase in brain $\beta$-HB levels in 3-day-fasted vs. nonfasted status correlated with the increase in plasma $\beta$-HB (mM) (29), suggesting that adaptation begins as early as 3 days of ketosis. Our results, together with others, suggest that the level and duration of ketosis regulate the protein expression of MCT-1 at the BBB and that a doubling of the $\beta$-HB extraction fraction relative to plasma $\beta$-HB concentration appears to be reflective of the upregulation in MCT-1.

Although altered blood flow associated with ketosis has been reported in some models (13, 21), this was not the case in our rat study. The increase in $\beta$-HB influx across the BBB was not a result of changes in blood flow. Regional brain blood flows measured in this study were comparable to those previously reported using the blood flow-indicator method (16, 17, 35, 44). These inconsistencies may be partly due to the process (acute vs. chronic) by which ketosis is induced, such as diet, fasting, or exogenous administration of ketones.

In an acute PET study of hyperketonemia (blood ketones 2.2 mM) in humans, global blood flow was reported to increase 39% relative to baseline (before ketone body infusion) with a fivefold increase in ketone uptake by brain and an equivalent reduction in glucose metabolism, reflecting the immediate uptake of ketones at the BBB and utilization by the brain (13). These results suggest that the increase in $\beta$-HB uptake is most likely related to the increase in blood ketones, as well as increased delivery (via increased blood flow). On the basis of our data (40-fold increase in influx with a 20-fold increase in...
plasma β-HB (4.0 mM)], blood ketone levels of 2.2 mM would predict a 10-fold increase in β-HB blood-to-brain influx, rather than a 5-fold increase, as reported in the human study. A twofold difference (5 vs. 10) may be the result of the lack of upregulation of MCT at the BBB in an acute condition, suggesting that an adaptive process occurs with chronic ketosis, such as that induced by diet.

To further explore whether the increase in β-HB influx was reflective of the upregulation in MCT-1, the functional role of MCT regulation at the BBB was evaluated. First, the evaluation was based on the idea that the extraction fraction is more of a functional measure of absolute β-HB influx at the BBB, inasmuch as it is not dependent on the β-HB plasma concentration or syringe withdrawal rate. It is the ratio of tracers in the brain to the periphery and, thus, is more reflective of the fractional changes in influx of β-HB at the BBB, which is indicative of the changes in transporter protein regulation. Our data show that the cortical extraction fraction of β-HB in the KG group reached about twice that of the STD group. Second, using immunohistochemical and Western blot analyses, we measured MCT-1 protein regulation at the BBB and found consistently a threefold induction in the capillaries compared with the STD group. Thus the increase in extraction fraction as a result of ketosis corresponds with the increase in MCT-1 at the BBB. These results support the hypothesis that the increase in MCT-1 is functional.

An upregulation of MCT-1 is thought to reflect the brain’s reliance on ketones as an alternate energy substrate to glucose (4, 6, 18, 19, 23, 42). In support of these findings, such characteristics would be advantageous to ensure continuous delivery of energy substrate to the brain during glucose sparing, such as with fasting or starvation or when the dietary primary fuel source is derived from fat, e.g., a KG diet. The significant increase regional β-HB influx with no changes in blood flow or plasma glucose levels suggests that ketone bodies are made more readily available to brain for oxidation, especially to regions with high metabolic rates. This would be critical during an energy deficit or a metabolic crisis.

The 40% increase in cortical capillary density observed with ketosis and the threefold increase in the fraction of capillaries that stained for MCT-1 suggest that a significant amount of ketone bodies are made available for brain metabolism. The colocalization of MCT-1 and GLUT-1 indicates that MCT-1 is localized to the microvessels. The increased colocalization of MCT-1 and GLUT-1 in the KG group also reflects the increased availability of ketones (23), but the fraction of ketones consumed for ATP production remains unknown.

Ketone body utilization in adult ketotic rat brain may account for >6% of cerebral metabolic rate of O2. We estimated that ∼35% of ATP production could come from the oxidation of ketones that are made available through its transport. Assuming the complete oxidation of β-HB and glucose, we based our estimate on the measured β-HB influx (0.4 μmol·g⁻¹·min⁻¹) and the influx for glucose (0.8 μmol·g⁻¹·min⁻¹), as previously reported (16). This is similar to β-HB and glucose influx projected for humans under chronic conditions of ketosis. In addition, in the KG group, the increase in GLUT-1 content measured in microvessels was three times greater than that measured in brain slices. These results suggest that the upregulation of GLUT-1 also occurred along the capillary.

The establishment of new microvascular patterns through angiogenic response to ketosis in rat brain is similar to hypoxia-induced angiogenesis (15). The link between angiogenesis and ketosis in unknown and was unexpected. One explanation may be elevated tissue levels of hypoxia inducible factor-1α, a transcription factor that mediates adaptive responses to hypoxia (unpublished data). Ketones, such as β-HB, may act as metabolic regulators related to the stabilization of hypoxia inducible factor-1α under normoxic conditions (22).

In conclusion, maintenance of rats on the KG diet results in a physiologically meaningful increase in plasma β-HB. This increase in plasma ketone bodies, together with the increase in extraction fraction and capillary density, indicates that a significant flux of substrates (reducing equivalents) is available for brain energy metabolism. Increased ketone availability, even in the presence of adequate glucose availability, leads to an upregulation of MCT-1 and an increase in β-HB influx. Whether there is an increase in net flux (ketone consumption for ATP production) with a decrease in glucose consumption remains to be determined.

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