Chapter 28
Diet-Induced Ketosis Protects Against Focal Cerebral Ischemia in Mouse

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Abstract Over the past decade we have consistently shown that ketosis is neuroprotective against ischemic insults in rats. We reported that diet-induced ketotic rats had a significant reduction in infarct volume when subjected to middle cerebral artery occlusion (MCAO), and improved survival and recovery after cardiac arrest and resuscitation. The neuroprotective mechanisms of ketosis (via ketogenic diet; KG) include (i) ketones are alternate energy substrates that can restore energy balance when glucose metabolism is deficient and (ii) ketones modulate cell-signalling pathways that are cytoprotective. We investigated the effects of diet-induced ketosis following transient focal cerebral ischemia in mice. The correlation between levels of ketosis and hypoxic inducible factor-1alpha (HIF-1α), AKT (also known as protein kinase B or PKB) and 5′ AMP-activated protein kinase (AMPK) were determined. Mice were fed with KG diet or standard lab-chow (STD) diet for 4 weeks. For the MCAO group, mice underwent 60 min of MCAO and total brain infarct volumes were evaluated 48 h after reperfusion. In a separate group of mice, brain tissue metabolites, levels of HIF-1α, phosphorylated AKT (pAKT), and AMPK were measured. After feeding a KG diet, levels of blood ketone bodies (beta-hydroxyburyrate, BHB) were increased. There was a proportional decrease in infarct volumes with increased blood BHB levels (KG vs STD; 4.2 ± 0.6 vs 7.8 ± 2.2 mm^3, mean ± SEM). A positive correlation was also observed with HIF-1α and pAKT relative to blood BHB levels. Our results showed that chronic ketosis can be induced in mice by KG diet and was neuroprotective

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against focal cerebral ischemia in a concentration dependent manner. Potential mechanisms include upregulation of cytoprotective pathways such as those associated with HIF-1α, pAKT and AMPK.

Keywords Ketogenic diet • Ischemia-reperfusion injury • HIF-1α, AKT • Neuroprotection.

1 Introduction

The ketogenic diet (KG) is a high-fat, very low-carbohydrate diet which results in hepatic production of ketone bodies due to elevated beta-oxidation of fats by the liver [1]. Ketone bodies (beta-hydroxybutyrate and acetoacetate; BHB, AcAc) are well utilized by brain as an energy substrate, especially during glucose sparing conditions, such as with long-term fasting or chronic feeding of a KG diet [2, 3]. Ketosis results in elevated blood ketone bodies which are alternate energy substrates to glucose and are known to be well utilized by brain. The KG diet is a well-established, non-pharmacological approach to treating drug-resistant epilepsy in children [4] and has shown promise in treating other neurological conditions such as Alzheimer and stroke. Ketones are also beneficial substrates during metabolic derangements of glucose metabolism such as with ischemia reperfusion injury induced oxidative stress [5]. The metabolic adaptation to chronic ketosis, as well as the mechanistic actions of ketosis in brain (globally and cellular) are multifactorial and not well understood. This study focused on investigating the potential mechanisms associated with hypoxic inducible factor-1alpha (HIF-1α) and neuroprotection in diet-induced ketotic mice. In mice pre-conditioned with a KG diet for 4 weeks, the effect of ketosis on brain focal cerebral ischemia (via reversible middle cerebral artery occlusion; MCAO) was investigated. Additionally, metabolic analysis of concentrations of energy metabolites and levels of HIF-1α, AKT, and 5′ AMP-activated protein kinase (AMPK) were also determined using mass-spectrometry and Western Blot methods.

2 Methods

2.1 Animals

Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University (CWRU). Male Blk6 Mice (11 weeks old) were fed either KG (high fat, carbohydrate restricted) or standard lab-chow (STD) diets for 4 weeks before ischemia experiments or tissue collections [6]. Mice were maintained on a 12:12 light-dark cycle with their diets and water available ad libitum. Diet Protocols: ketogenic (KG; 89.5 fat %, 10.4 protein %, 0.1

CHO %; Research Diets, New Brunswick, NJ, USA, diet) and standard (STD; 27.5 fat %, 20.0 protein%, 52.6, provided by the CWRU animal facility). Weekly blood ketone body (BHB) concentrations were analysed using a keto-meter (Precision Xtra, Abbott, Alameda, CA, USA) from a small blood sample taken from the tail and their body weights were monitored pre- and post-diet treatment.

2.2 Middle Cerebral Artery Occlusion (MCAO)

Transient focal ischemia using a mouse MCAO model [7]: MCA mice underwent 60 min of MCAO and reperfusion. To ensure consistent and successful blockage of MCA, we monitored ischemia in all of our animals by Laser Doppler flowmetry (PeriFlux System 5000). Mice were perfused transcardially and the total infarct volumes were evaluated by Giemsa staining 48 h after reperfusion. The infarct areas are quantified using the NIH ImageJ software.

2.3 Western Blot Analysis

The analysis of HIF-1α, and related cell signalling targets such as AKT and AMPK, were measured by Western Blot analysis. Proteins (20 μg) from homogenized whole brain tissues were separated on a 12% gel, then transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% bovine serum albumin (BSA), and incubated with primary antibody overnight. Antibodies: HIF-1α (R&D, [1:2000]), AKTtot (CST, [1:2000]), pAKTser473 (CST, [1:2000]), pAKTthr308 (CST, [1:500]), and HSC70 (loading control, Santa Cruz, [1:5000]). Following incubation with secondary antibodies (1:15,000), proteins were detected by chemiluminescence, and densitometry was performed using Image J software [8].

2.4 Metabolic Panels: GCMS-Based Analysis

Targeted metabolic profiling by GCMS (gas chromatography-MS) analysis included measurements of citric acid cycle intermediates (CAC; absolute concentrations; μmol/g tissue) collected from fresh frozen brain tissue homogenates of mice fed either KG or STD diets [9]; the GC-MS analysis of CAC intermediates were analysed as their trimethylsilyl (TBDMS; Regis) derivatives on an Agilent 5973 N-MSD equipped with an Agilent 6890 GC system coupled to a DB-17MS capillary column (30 m × 0.25 mm × 0.25 μm) and operated in electron impact ionization mode [10].

2.5 Statistical Analysis

All values were presented as mean ± SEM. Statistical analyses were performed using SPSS v 20.0 for Windows. The comparison between any two groups was analysed with a t-test for paired sample, two-tailed. Significance was considered at the level of $p < 0.05$.

3 Results

3.1 Diet-Induced Ketosis on Infarct Volume Following MCAO in Mice

After 4 weeks of the KG diet, plasma ketone bodies (beta-hydroxyburyrate, BHB; mM) were increased (range 1.1–2.6) in the KG mice. The infarct volumes were decreased in the KG group (n = 7) compared to the STD group (n = 4), (4.2 ± 0.6 vs 7.8 ± 2.2 mm^3, mean ± SEM, p = 0.16) in a concentration-dependent manner, as there was a proportional decrease in infarct volume with increased blood BHB levels (Fig. 28.1).

3.2 Diet Induced Ketosis on HIF-1α Accumulation, AKT Activation, AMPK and Metabolites

HIF-1α levels were measured in cortical brain homogenates of KG (n = 5) and STD (n = 5) diet mice by Western Blot analysis. The HIF-1α levels increased significantly in the KG diet group (Fig. 28.2) compared to baseline levels of the STD diet group. In Fig. 28.3, the levels of the blood BHB was graphed against the abundance of protein targets detected. There was a positive correlation between the protein targets of HIF-1α and AKT (phosphorylation of ser473, thr308), and the circulating BHB concentrations (mM) in mice fed a KG diet for 4 weeks. AKT_{total} was not significantly different between the two groups. There was an insignificant positive correlation between HIF-1α and blood BHB (Fig. 28.3a). While the phosphorylation of both the serine and threonine sites of AKT showed a strong positive correlation to the level of blood BHB, the serine site showed a statistically significant correlation (Fig. 28.3b). Protein levels of AMPK (phosphorylation and total) were also

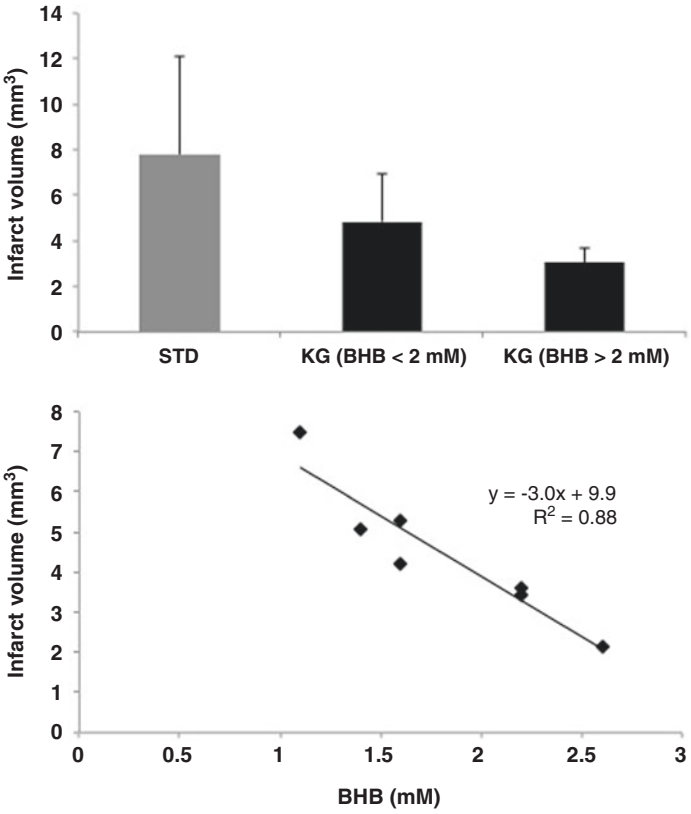

Fig. 28.1 *Upper panel*: infarct volume (mm^3) in the STD and KG groups at 48 h reperfusion following 60 min middle cerebral artery occlusion (MCAO). *Lower panel*: correlation of infarct volume with blood BHB levels, there was a decreased infarct volume with increased blood BHB levels (STD group: n = 4; KG group; n = 7)

significantly increased in the KG diet group compared to the STD group (Fig. 28.3c, d). Additionally, there was a significant correlation of the $pAKT_{thr}$ and the CAC intermediates (fumarate and malate) and BHB (tissue and plasma), Fig. 28.4. Although there were no significant findings with the other metabolites measured (succinate, citrate, aspartate, 2-hydroxyglutarate, GABA), their concentrations trended higher in the KG diet group compared to STD group (data not shown).

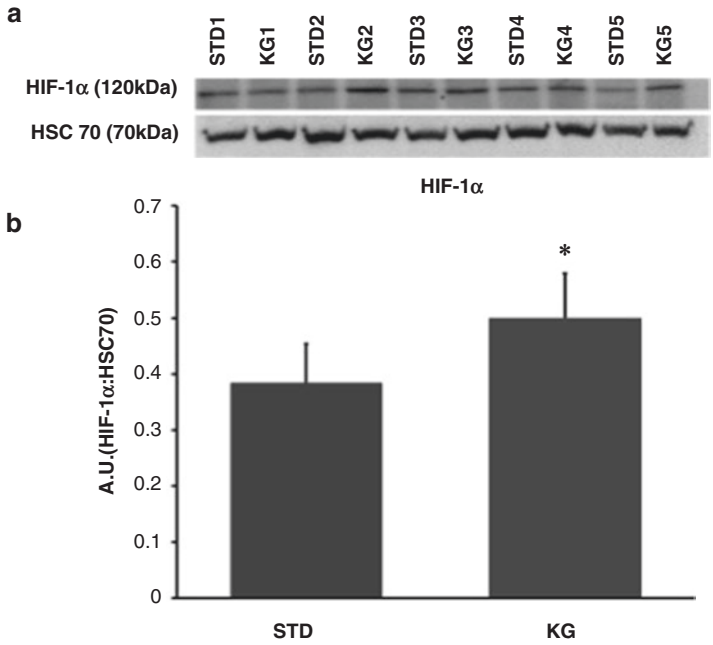

Fig. 28.2 KG diet upregulates HIF-1α protein. (**a**) Western Blot analysis of HIF-1α. (**b**) HIF-1α protein levels were normalized to HSC 70 loading control. HIF-1α was significantly higher in KG mice. The values presented are the mean ± S.E.M (n = 5). *Denotes statistical significance ($p < 0.05$)

4 Discussion

The mechanism through which KG confers neuroprotection is still largely unknown, but has been thought to be through the fact that ketones are alternate energy substrates to glucose [2–6], especially under conditions when glucose metabolism is impaired such as with ischemia reperfusion injury [5]. More recent studies have implicated KG in modulating cell-signaling pathways that are cytoprotective. The primary signaling pathways associated with cyto-protection include HIF-1α, AKT, and AMPK and these pathways are known to have overlapping signaling targets. Neuroprotective properties of ketosis may be through HIF-1α, a primary constituent associated with hypoxic angiogenesis and a regulator of neuroprotective responses [11, 12]. Our results demonstrate that the diet-induced ketotic mice exhibited significant upregulation in HIF-1α and AMPK phosphorylation, as well as neuroprotection in KG mice exposed to MCAO. These changes correlated strongly with blood BHB levels. Circulating BHB levels correlated with pAKT protein targets, suggesting a dose-dependent effect on the activity of signalling targets that are both localized and systemic. The association between pAKT and metabolites (e.g. fumarate and malate) also suggested that the neuroprotective properties of ketosis may be

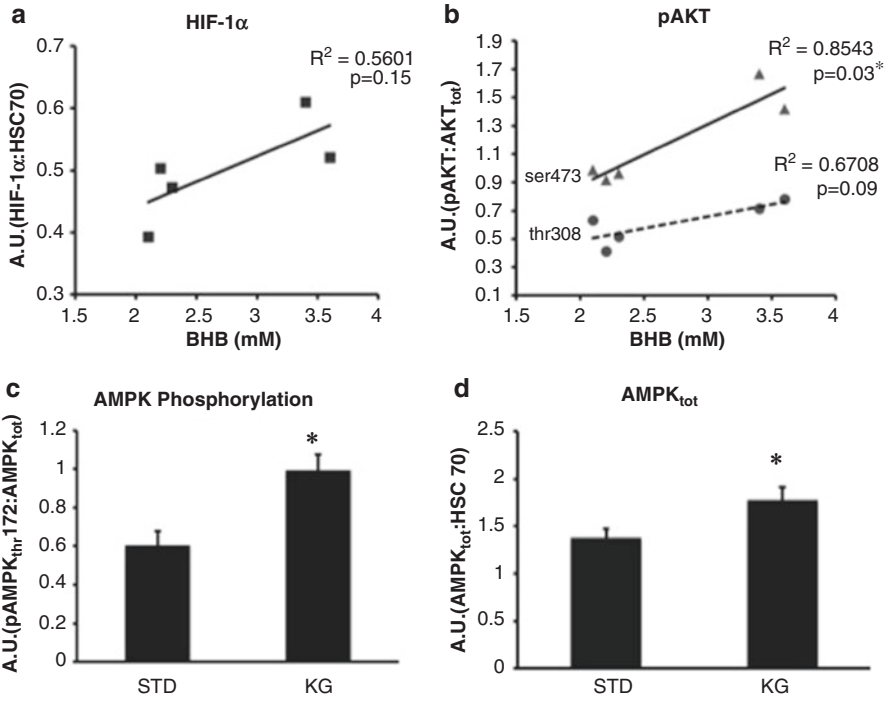

Fig. 28.3 Correlation between protein targets and circulating BHB concentrations in mice fed a ketogenic diet for 4 weeks. (**a**) Positive correlation between HIF-1α and plasma BHB. (**b**) Phosphorylation of both the serine and threonine sites of AKT showed positive correlation to blood BHB levels. (**c**) AMPK phosphorylation was significantly increased in KG group. (**d**) AMPK total. *indicates significance ($p < 0.05$; n = 5 per group)

through the modulation of energetics or redox state of the cell. Our work represents a novel finding with respect to post-translational regulation of a family of cytoprotective proteins that are regulated in concentration manner related to the degree of ketosis. Such an adaptive response to ketosis implies a greater sensitivity of cellular signalling pathways to alterations in circulating ketone bodies than previously reported.

In summary, our results showed that ketosis can be induced in mice by a KG diet and is neuroprotective against focal cerebral ischemia in a concentration-dependent

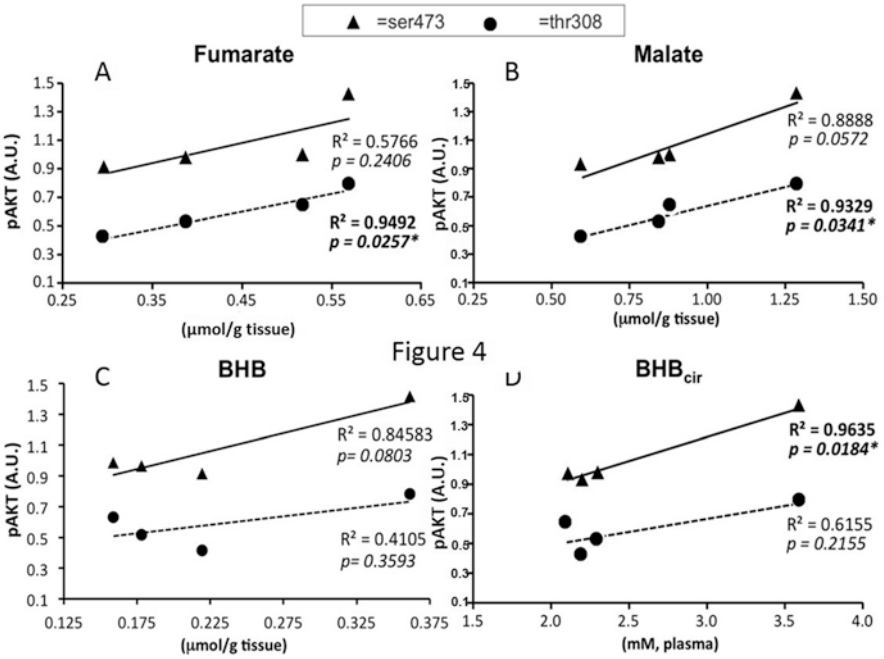

Fig. 28.4 Diet-induced activation of pAKT correlates with metabolic response and level of ketosis. *Upper panels*: citric acid cycle intermediates, fumarate and malate, showed a positive correlation with protein levels of pAKT (ser473 and thr308). *Lower panels*: positive correlation between pAKT and BHB levels as measured in both blood and brain tissue. *indicates significance ($p < 0.05$), n = 5 per group

manner. Potential mechanisms include upregulation of cellular salvation pathways, such as those associated with HIF-1α, AKT and AMPK.

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