



Review article

Do ketone bodies mediate the anti-seizure effects of the ketogenic diet?

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ABSTRACT

Although the mechanisms underlying the anti-seizure effects of the high-fat ketogenic diet (KD) remain unclear, a long-standing question has been whether ketone bodies (i.e., β -hydroxybutyrate, acetoacetate and acetone), either alone or in combination, contribute mechanistically. The traditional belief has been that while ketone bodies reflect enhanced fatty acid oxidation and a general shift toward intermediary metabolism, they are not likely to be the key mediators of the KD's clinical effects, as blood levels of β -hydroxybutyrate do not correlate consistently with improved seizure control. Against this unresolved backdrop, new data support ketone bodies as having anti-seizure actions. Specifically, β -hydroxybutyrate has been shown to interact with multiple novel molecular targets such as histone deacetylases, hydroxycarboxylic acid receptors on immune cells, and the NLRP3 inflammasome. Clearly, as a diet-based therapy is expected to render a broad array of biochemical, molecular, and cellular changes, no single mechanism can explain how the KD works. Specific metabolic substrates or enzymes are only a few of many important factors influenced by the KD that can collectively influence brain hyperexcitability and hypersynchrony. This review summarizes recent novel experimental findings supporting the anti-seizure and neuroprotective properties of ketone bodies.

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Contents

1. Introduction	233
2. Evidence for the anti-seizure activity of ketone bodies in <i>in vivo</i> seizure models	234
3. Evidence of inhibitory properties of ketone bodies <i>in vitro</i>	236
4. Molecular targets	237
4.1. Mitochondria	237
4.2. Neurotransmitter systems	238
4.3. ATP-sensitive potassium (K_{ATP}) channels	238
4.4. Histone (lysine) deacetylases and gene regulation	239
4.5. Anti-inflammatory effects	239
5. Summary	239
Acknowledgements	240
References	240

1. Introduction

The ketogenic diet (KD) is a high-fat, low-carbohydrate and adequate-protein formulation that has been used for nearly a

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century to treat medically intractable epilepsy. Although the mechanisms underlying the KD's clinical effects remain unclear (Rho and Stafstrom, 2012; Rogawski et al., 2016), it remains controversial whether any or all of the major ketone bodies (e.g., beta-hydroxybutyrate, acetoacetate and acetone) produced by the liver are directly responsible for the KD's anti-seizure profile. One reason for this persistent uncertainty is the clinical observation that blood ketone (i.e., β -hydroxybutyrate) levels do not correlate well with seizure control (Kossoff et al., 2009; Kossoff and Rho, 2009; but see Gilbert et al., 2000; van Delft et al., 2010), although local ketone levels at the neuronal or synaptic level may be a more accurate reflection of ketone effects on excitability (Stafstrom, 2004). Further, another diet that has been used successfully to treat patients with medically intractable epilepsy, the low glycemic index treatment (LGIT), does not induce systemic ketosis (Muzykewicz et al., 2009). While definitive evidence in this regard is not yet forthcoming, recent clinical data indicate that ketone bodies (specifically, β -hydroxybutyrate) may yet be relevant to an anti-seizure effect (Buchhalter et al., 2017). In contrast, experimental data are more compelling, with recent studies highlighting pleiotropic actions of β -hydroxybutyrate and novel molecular targets (Puchalska and Crawford, 2017). While some of these mechanistic observations have yet to be firmly and causally linked to an attenuation of seizure activity, the scientific rationale for both ketone-induced anti-seizure and neuroprotective effects has grown substantially over the past few years (Gano et al., 2014; Puchalska and Crawford, 2017). This review summarizes the evidence for ketone bodies as important contributors to ketogenic diet effects in the clinical setting (see Table 1, Fig. 1).

2. Evidence for the anti-seizure activity of ketone bodies in *in vivo* seizure models

Since the introduction of the KD and the hypothesis that ketone bodies are responsible for its therapeutic effects, there has been a relative paucity of *in vivo* studies demonstrating the therapeutic efficacy of ketones against seizures. However, since the turn of the 21st century, evidence has been quickly accumulating, supporting the notion that ketone bodies can indeed contribute to seizure control. Initial screening for anti-seizure activity usually begins with determination of dose-dependent protection of an acutely administered compound against an induced seizure in normal (i.e., non-epileptic) animals. In the case of ketone bodies, the first documented testing began with Keith (1933, 1935) who found that acetone and acetoacetate, but not β -hydroxybutyrate, protected rabbits against seizures induced by thujone, a constituent of wormwood oil and a known antagonist of γ -aminobutyric acid, type A (GABA_A) receptors (Höld et al., 2000).

Nearly seventy years passed before interest in ketone bodies was revived. Acetone and acetoacetate, but not β -hydroxybutyrate, protected against sound-induced seizures in the Frings audiogenic seizure-susceptible mouse model (Rho et al., 2002). Shortly thereafter, acetone was found to increase the seizure threshold of rats in multiple models of seizure induction, including the maximal electroshock test which models tonic-clonic seizures, the pentylenetetrazol test which models typical absence seizures, the amygdala kindling test which models complex partial (more recently termed focal with impaired awareness) seizures with bilateral spread, and the AY-9944 test which models chronic atypical absence seizures (Likhodii and Burnham, 2002; Likhodii et al., 2003). Collectively, these data suggest that acetone has a broad-spectrum anti-seizure profile similar to the clinical experience with the KD. Effects of acetone in the pentylenetetrazol test and electroshock test have been confirmed, and extended to protection against tonic seizures in the 4-aminopyridine (4-AP) test and

reduced seizure severity during lithium-pilocarpine status epilepticus (Gasior et al., 2007; Inoue et al., 2009; Hasebe et al., 2010). Similar to acetone, acetoacetate reduced seizures induced by intrahippocampal 4-AP infusion in rats (Juge et al., 2010). Furthermore, acetoacetate and its analog 2-phenylbutyrate decreased hippocampal seizure activity in the intrahippocampal kainate model of chronic epilepsy (Kadowaki et al., 2017).

Recently, ketone esters have been investigated as a potential “pro-drug” capable of sustained elevation of ketone bodies (D'Agostino et al., 2013). The *R,S*-1,3-butanediol acetoacetate diester (BD-AcAc2) resulted in elevated blood acetone, acetoacetate and β -hydroxybutyrate levels in rats and increased the latency to hyperbaric oxygen-induced seizures. In contrast, 1,3-butanediol raised only blood β -hydroxybutyrate levels and failed to affect seizure latencies. Single or repeated dosing of BD-AcAc2 has been further demonstrated to increase the threshold of pentylenetetrazole-induced seizures in rats (Viggiano et al., 2015, 2016) and audiogenic- and kainate-induced seizures in a mouse model of Angelman syndrome (Ciarlone et al., 2016).

Based on the aforementioned studies, it would appear that β -hydroxybutyrate does not contribute to the anti-seizure efficacy of the ketogenic diet. However, three recent studies indicate that β -hydroxybutyrate may yet play a role. In an effort to determine if the higher endogenous β -hydroxybutyrate levels in suckling neonates confers seizure protection, Minlebaev and Khazipov (2011) performed a series of depth electrode experiments on postnatal day 5–9 non-anesthetized rat pups. Inhibiting ketogenesis had no effect on seizures provoked by a single flurothyl exposure. However, seizures during a second exposure were exacerbated. This effect was reversed with administration of exogenous β -hydroxybutyrate, suggesting a reduction of hyperexcitability and a direct role of β -hydroxybutyrate in raising the seizure threshold in neonates (Minlebaev and Khazipov, 2011). The second study investigated the effects of β -hydroxybutyrate in the betamethasone-NMDA model of infantile spasms (Yum et al., 2015). A single administration of β -hydroxybutyrate failed to affect the spasms, but repeated injections over three days increased the latency and decreased the number of spasms. Furthermore, this anti-seizure effect was enhanced with repeated bouts of NMDA-triggered spasms (Yum et al., 2015). The third study involved chronic infusion of β -hydroxybutyrate via osmotic minipumps over a two-week period in a genetic model of epilepsy and reported that β -hydroxybutyrate reduced spontaneous recurrent seizures similar to a ketogenic diet (Kim et al., 2015a,b). Collectively, these studies suggest that the anti-seizure effects of β -hydroxybutyrate *in vivo* may have been missed in previous experiments due to limited, single dosing and/or the use of acute seizure models rather than models replicating aspects of spontaneous recurrent seizures.

In pediatric and adult patients or animals clinically used KDs generally raise plasma levels of ketone bodies to between 1 and 10 mM (Gilbert et al., 2000; van Delft et al., 2010; Simeone et al., 2016; 2017). Importantly, all of the *in vivo* studies described above used doses that would raise plasma ketones to within this range (e.g., an intraperitoneal injection of 10 mmol/kg β -hydroxybutyrate raises β -hydroxybutyrate to 5–7 mM, injection of 8 mmol/kg acetone raises acetone to 4–6 mM and intragastric administration of the pro-drug BD-AcAc2 raises acetone to 1 mM, β -hydroxybutyrate to 4 mM and acetoacetate to 4 mM; Eiger et al., 1980; Likhodii and Burnham, 2002; Likhodii et al., 2003; D'Agostino et al., 2013; Yum et al., 2015). Moreover, those studies performing dose response experiments calculated anti-seizure ED₅₀'s that would result in this mM range.

Before delving into the *in vitro* evidence of ketone-mediated

Table 1
Ketone Body efficacy in *in vivo* and *in vitro* models of seizures and hyperexcitability.

In vivo	Acetone	AcAc	BHB	References
<i>Induced Seizures</i>				
Thujone	+	+	–	a, b
Audiogenic	+	+(BD-AcAc2, AS mice)	–	c, m
MES	+	N.D.	N.D.	d
PTZ	+	+(BD-AcAc2)	N.D.	d, e, g, k, l
Amygdala Kindling	+	N.D.	N.D.	d
AY-9944	+	N.D.	N.D.	d
4-AP	+	+	N.D.	e, h
Li-pilo SE	+	N.D.	N.D.	f
Hyperbaric Oxygen	N.D.	+(BD-AcAc2)	-(1,3-butanediol)	j
Kainate	N.D.	+(BD-AcAc2, AS mice)	N.D.	m
Repeated flurothyl	N.D.	N.D.	+(rat neonates)	n
<i>Spontaneous Seizures</i>				
Intrahippocampal Kainate	N.D.	+	N.D.	i
betamethasone-NMDA	N.D.	N.D.	+	o
<i>Kcna1</i> -null mice	N.D.	N.D.	+	p
In vitro	Acetone	AcAc	BHB	References
<i>Cellular and population excitability</i>				
mEPSCs	N.D.	+	N.D.	h
Field Potentials	N.D.	–	–	q, p, s
Population Spikes	N.D.	–	–	q, s
LTP	N.D.	–	–	p, r, s
<i>Mitochondrial mediated synaptic dysfunction</i>				
ETC inhibitors	N.D.	+	+	p, r, v
ROS	N.D.	+	+	p, u, v
<i>Induced Seizure-like events in slices</i>				
4-AP	N.D.	N.D.	–	w
Low Mg ²⁺	N.D.	N.D.	–	t, w
PTZ	N.D.	–	–	t
bicuculline	N.D.	N.D.	–	
High frequency stimulation	N.D.	N.D.	–	w
OGD	N.D.	N.D.	+	w
<i>Kcna1</i> -null mice	N.D.	+	+	p

Abbreviations: AcAc, acetoacetate; BHB, β -hydroxybutyrate; MES, maximal electroshock; PTZ, pentylenetetrazole; 4-AP, 4-aminopyridine; Li-pilo, lithium pilocarpine; SE, status epilepticus; HFS, high-frequency stimulation; OGD, Oxygen-glucose deprivation; N.D., not determined; +, attenuation effect; –, inactive; BD-AcAc2, *R,S*-1,3-butanediol acetoacetate diester; AS, Angelman syndrome; mEPSC, miniature excitatory post-synaptic current; LTP, long-term potentiation; ETC, electron transport chain; ROS, reactive oxygen species.

- a. Keith, 1933.
 b. Keith, 1935.
 c. Rho et al., 2002.
 d. Likhodii et al., 2003.
 e. Gasior et al., 2007.
 f. Inoue et al., 2009.
 g. Hasebe et al., 2010.
 h. Juge et al., 2010.
 i. Kadowaki et al., 2017.
 j. D'Agostino et al., 2013.
 k. Viggiano et al., 2015.
 l. Viggiano et al., 2016.
 m. Ciarlone et al., 2016.
 n. Minlebaev and Khazipov, 2011.
 o. Yum et al., 2015.
 p. Kim et al., 2015a,b.
 q. Thio et al., 2000.
 r. Kimura et al., 2012.
 s. Youssef, 2015.
 t. Chang et al., 2016.
 u. Maalouf and Rho, 2008.
 v. Kim et al., 2010.
 w. Samoilova et al., 2010.

inhibition of network excitability and mechanistic studies, it is important to briefly address how plasma ketone levels are related to brain ketone levels as this informs the physiological relevance of *in vitro* experiments. Ketone bodies enter the brain via monocarboxylate transporters which are expressed more highly in suckling than mature animals (Nehlig, 2004), although dietary ketosis may increase expression in adults (Leino et al., 2001).

Acetoacetate and β -hydroxybutyrate exist in a stoichiometric relationship (Owen et al., 1967). The ratio of acetoacetate to β -hydroxybutyrate is approximately 1:3 in plasma of fasting or KD-treated humans and 1:2 in cerebral spinal fluid (CSF) of KD-treated humans and brain of KD-fed rats (Balasse and Fery, 1978; Devivo et al., 1978; Nordli and De Vivo, 1997). Magnetic resonance spectroscopy (MRS) studies in KD-treated epileptic children

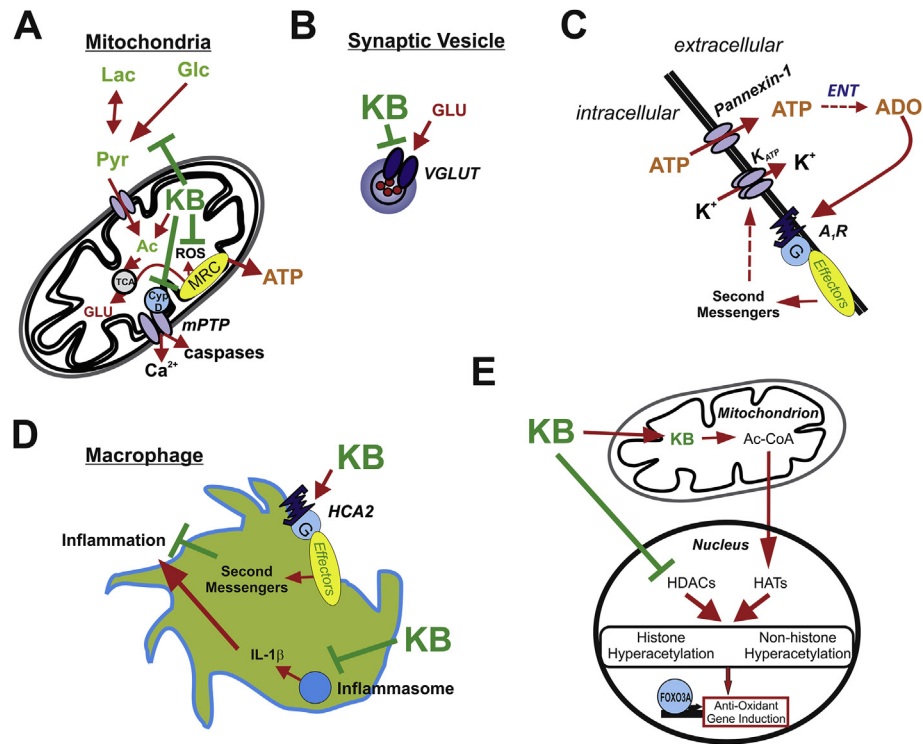


Fig. 1. Illustrations depicting potential mechanisms underlying ketone body-mediated attenuation of CNS hyperexcitability and neuroprotection. (A) Ketone bodies (KB) may enhance ATP production by providing acetyl-CoA (Ac) and inhibit production of reactive oxygen species (ROS) and the mitochondrial permeability transition (mPT) pore, thereby protecting the cell against oxidative injury and preventing excessive release of calcium. (B) KB may inhibit vesicular glutamate transporters (VGLUT), decreasing the amount of glutamate loaded in vesicles and reducing the size of glutamate quanta released during synaptic transmission. (C) KB-mediated increases in intracellular ATP and subsequent release through pannexin channels lead to adenosine (ADO) synthesis via ectonucleotidases (ENT) in the extracellular space. ADO in turn binds to inhibitory adenosine type 1 receptors (A_1R s) which are coupled to the indirect opening of K_{ATP} channels. (D) KB activate HCA2 receptors and inhibit the assembly of the NLRP3 inflammasome; thus, KB attenuate inflammatory mediators produced by infiltrating macrophages. (E) KB also promote histone and non-histone hyperacetylation by increasing acetyl-CoA, a substrate for histone acetyltransferases (HATs), and directly inhibiting histone deacetylases (HDACs) – with the end result of increasing endogenous anti-oxidants (among other actions).

and fasted adults have demonstrated that dietary ketosis dramatically increases brain β -hydroxybutyrate, and that brain and plasma concentrations are linearly related with brain β -hydroxybutyrate being approximately 26% of plasma concentrations (Shen et al., 1998; Pan et al., 2000). This is in rough agreement with the relationship found between brain and plasma of KD-fed mice (18%; Samala et al., 2011). However, microdialysis in the hippocampus of KD-fed mice indicate that β -hydroxybutyrate in extracellular fluid is 5% of the plasma concentration. In contrast, acetone has been found to have a 1:1 relationship between blood and CSF (Likhodii and Burnham, 2002; Likhodii et al., 2003). Collectively, these findings suggest that the relevant physiological concentrations for β -hydroxybutyrate, acetoacetate and acetone range from 0.05 to 2.5 mM, 0.025–1.25 mM and 1–10 mM, respectively. Therefore, in the following sections concerning *in vitro* studies regarding ketone body effects on cellular excitability and potential mechanisms, we make a point to state whether concentrations used fall within physiological relevance or, even better, whether the study involved full concentration responses spanning physiological levels. Studies using concentrations outside the physiological range are also presented, but should be interpreted with caution.

3. Evidence of inhibitory properties of ketone bodies *in vitro*

In general, ketone body effects on neuronal and network excitability have been investigated using acutely prepared hippocampal slices from normal mice or rats. Thus far, the results have been inconsistent. Juge et al. (2010) reported that a high concentration of acetoacetate (10 mM) reduced mEPSC frequency and

amplitude in CA1 neurons using whole-cell patch clamp techniques. However, using extracellular recording conditions, multiple groups have found that physiologically relevant concentrations of acetoacetate (1–3 mM) and β -hydroxybutyrate (1–3 mM) exert no effects on evoked field potentials, population spikes or long-term potentiation (Thio et al., 2000; Kimura et al., 2012; Kim et al., 2015a,b; Youssef, 2015). Similarly, seizure-like events (SLEs) induced by application of pentylenetetrazole or low-magnesium were not reduced when exposed to high concentrations of either acetoacetate (10 mM) or β -hydroxybutyrate (10 mM) (Chang et al., 2016). In contrast, pretreatment with acetoacetate and β -hydroxybutyrate (co-application of 1 mM each) prevented synaptic dysfunction due to mitochondrial electron transport chain inhibitors or exogenously applied reactive oxygen species (ROS) (Maalouf and Rho, 2008; Kimura et al., 2012; Kim et al., 2010, 2015a,b). Further, 0.5–10 mM β -hydroxybutyrate restored synaptic function during glucose deprivation, but only in slices from rats postnatal day 30 or younger; however, β -hydroxybutyrate provided protection against glucose deprivation-mediated morphological damage at every age (Izumi et al., 1998).

The root cause(s) of these *in vitro* inconsistencies may reflect challenges observed *in vivo*, e.g. duration of exposure to ketone bodies may be too short. In an attempt to circumvent this limitation of acute slices, two studies used organotypic hippocampal slice cultures. Samoilova et al. (2010) applied 10 mM β -hydroxybutyrate to slice cultures for 3 days and provoked SLEs under five different conditions: 4-aminopyridine (4-AP), low-magnesium, bicuculline, high-frequency stimulation and oxygen-glucose deprivation (OGD). β -hydroxybutyrate did not affect the properties of SLEs under any

of these provocative manipulations except the metabolic insult of OGD. Specifically, upon re-perfusion after OGD, β -hydroxybutyrate prevented the development of SLEs that are ordinarily observed in >80% of slice cultures (Samoilova et al., 2010). Using a slightly different paradigm, Kim et al. (2015a,b) generated hippocampal slice cultures from epileptic *Kcna1*-null mice that developed spontaneous SLEs. Incubation with a cocktail of 1 mM acetoacetate and 5 mM β -hydroxybutyrate for two-weeks reduced the frequency, duration and intensity of the SLEs (Kim et al., 2015a,b). It is worth noting that acutely isolated mitochondria from *Kcna1*-null hippocampi are dysfunctional and likely contribute to the synaptic and network hyperexcitability underlying seizure genesis in this mouse model (Simeone et al., 2014a; Kim et al., 2015a,b).

Overall, these studies with specific ketone bodies mirror *in vitro* experiments with acute hippocampal slices from mice and rats fed a KD. Specifically, a KD did not affect synaptic function in normal rats or prevent low-magnesium provoked hyperexcitability (Stafstrom et al., 1999; Bough et al., 2006), but it did prevent synaptic depression by glucose-deprivation (Bough et al., 2006). Furthermore, unprovoked network and synaptic hyperexcitability of slices from KD treated epileptic mice and rats were significantly dampened (Stafstrom et al., 1999; Simeone et al., 2014b).

Even though there are experimental design and technical differences among the cited studies, taken together, it is reasonable to conclude that ketone bodies have minimal or no effect on normal synaptic and network excitability, but do reduce the consequences of diverse metabolic insults. Moreover, ketone bodies may be unable to overcome hyperexcitability due to widespread, near-complete modulation of receptor and ion channel systems (i.e., non-selective inhibition of GABA_A receptors with bicuculline or pentylentetrazole, non-selective block of potassium channels with 4-AP, and disinhibition of NMDA receptors with low magnesium). However, it should be noted that in these experiments, concentrations of modulators that elicit maximum effects were used. Perhaps performing concentration-response experiments with these pro-convulsant modulators may reveal meaningful ketone body effects.

Based on the above, it is tempting to speculate why the KD is ineffective in some compliant patients. Perhaps a metabolic derangement plays a minimal role in the patient's specific epilepsy syndrome. Alternatively, genetic or epigenetic factors may significantly alter receptor or channel function. At this time, these hypotheses await experimental and clinical verification.

4. Molecular targets

The *in vivo* and *in vitro* studies discussed thus far provide solid evidence that ketone bodies do indeed exert anti-seizure effects. Although the primary determinants of neuronal excitability have traditionally focused on the major excitatory and inhibitory neurotransmitter systems in the brain (i.e., glutamate and γ -aminobutyric acid or GABA, respectively), recently there has been growing recognition of additional factors that influence neuronal excitability directly. These include mitochondrial health (their ability to produce ATP, buffer calcium and generate ROS), antioxidant systems, inflammatory mediators, and histone deacetylation status (which modifies transcription of genes). Here, we review the key studies supporting ketone body modulation of these diverse mechanisms.

4.1. Mitochondria

Several elegant studies have demonstrated that mitochondria regulate synaptic transmission via three mechanisms: (1) production of ATP, (2) generation of ROS, and (3) sequestration of cytosolic

calcium (Lee et al., 2007, 2012; Harris et al., 2012). Thus, any perturbation of mitochondrial health will send ripples of dysregulation across synaptic, neuronal and network activities. Moreover, prolonged excitotoxicity and concomitantly rising intracellular calcium levels can initiate the opening of the mitochondrial membrane permeability transition pore (mPTP), through which pro-apoptotic factors are released into the cytosol and which invariably lead to cell death. Stabilizing mitochondria could be a mechanism by which ketone bodies reduce seizure activity and cellular demise.

Krebs cycle: At the heart of a long-standing hypothesis concerning the role of ketone bodies in KD action is that they are a more efficient fuel source than glucose. β -hydroxybutyrate converts to acetoacetate via the enzyme β -hydroxybutyrate dehydrogenase and acetoacetate skips glycolysis, directly entering the Krebs cycle at the level of acetyl-CoA. Using nuclear magnetic resonance (NMR) techniques in rat hippocampal slices, Valente-Silva et al. (2015) reported that given equal amounts of substrate, ketone bodies (5 mM) out-compete glucose for neuronal acetyl-CoA by inhibiting glycolytic flux upstream of pyruvate kinase. A similar finding was made in brain homogenates from rats fed a KD using isotopomer mass spectrometry analysis (Zhang et al., 2015). In the heart, ketone body metabolism maximizes the redox difference between the mitochondrial NAD⁺/NADH couple of Complex I and the co-enzyme Q/QH₂ couple of Complex II, resulting in an increased proton gradient, enhanced potential for ATP synthesis and increases in the free energy of ATP hydrolysis. Collectively, these changes indicate that more energy is stored in ATP and released when ATP becomes ADP (Sato et al., 1995; Veech et al., 2001). Ketone body metabolism also decreases mitochondrial free radical production through at least two mechanisms: (1) decreasing the reduced form of co-enzyme Q (which normally reacts with O₂ to form the superoxide radical O₂⁻) and (2) increasing the ratio of [NADPH]/[NADP⁺] (which promotes glutathione reductase to reduce glutathione and restore antioxidant capacity) (Veech et al., 2001). These actions serve to maintain Complex I-driven mitochondrial respiration and ATP synthesis under stressful conditions (Maalouf et al., 2007; Kim et al., 2010).

Membrane Permeability Transition Pore (mPTP): mPTP is a massive channel (>1 nS conductance) that is believed to regulate the homeostatic status of mitochondria (Nicholls and Budd, 2000). Prolonged opening of the mPTP is promoted by excessive concentrations of calcium and/or ROS. The resultant permeability transition results in collapse of the mitochondrial membrane potential, inhibition of ATP production via uncoupling of the electron transport system from ATP synthase, mitochondrial swelling, and the release of calcium, ROS and pro-apoptotic proteins such as cytochrome c into the cytosol, which together can trigger cell-death (Sullivan et al., 2005). A series of studies over the past decade have demonstrated that acetoacetate and β -hydroxybutyrate (individual or co-application, 0.1–3 mM) can inhibit mPTP opening indirectly via decreased ROS levels as well as facilitate enhanced mitochondrial respiration, NADH oxidation and ATP production (Maalouf et al., 2007; Maalouf and Rho, 2008; Kim et al., 2007, 2010, 2015a,b). Ultimately these actions lead to decreased interaction of cyclophilin D (CypD), a key regulator of mPTP calcium sensitivity (Hurst et al., 2016), with mPTP, as ketone bodies were ineffective in mitochondria lacking the CypD subunit (Kim et al., 2015a,b). Furthermore, the importance of mPTP and CypD for the KD's anti-seizure effects were demonstrated when *in vivo* administration of atractyloside, a mPTP opener, blocked KD-induced reduction of spontaneous recurrent seizures in epileptic *Kcna1*-null mice, whereas NIM811, a selective CypD inhibitor, mimicked the KD with respect to attenuation of seizure activity (Kim et al., 2015a,b).

Bcl-2-associated Agonist of Cell Death (BAD): The mitochondrial

membrane potential depends on several factors, an important one being the balanced presence of anti-apoptotic Bcl-2 and Bcl-xl and pro-apoptotic Bad and Bax. Upon injury, Bad and Bax are dephosphorylated and translocate to mitochondria, bind Bcl-2 and Bcl-xl, and depolarize mitochondria. Significant depolarization of the mitochondrial membrane potential can lead to the release of pro-apoptotic factors such as cytochrome C, caspase 9 and caspase 3 (Youle and Strasser, 2008). The phosphorylation state of three serines, Ser112, Ser136 and Ser155, determine BAD function. Phosphorylation of Ser112 and Ser136 promotes binding of BAD to 14-3-3 proteins, sequestering BAD away from the mitochondrial membrane (Tan et al., 2000). The KD increased phosphorylation of BAD Ser136 and the interaction between BAD and 14-3-3, actions which may underlie its neuroprotective properties against kainic acid-induced status epilepticus (Noh et al., 2006). Similarly, *in vivo* administration of β -hydroxybutyrate increased 14-3-3 mRNA and phosphorylation of BAD Ser136 and decreased pulmonary apoptosis in a rat model of hemorrhagic shock (Jaskille et al., 2004).

Phosphorylation of Ser155 blocks binding of BAD to Bcl-xl (Tan et al., 2000), but also induces glucokinase binding and increases the rate of glycolysis (Danial et al., 2003). Thus, phosphorylated BAD Ser155 shifts metabolism in favor of glucose. Giménez-Cassina et al. (2012) hypothesized that dephosphorylated BAD Ser155 may confer preference for ketone body metabolism and raise seizure thresholds. Indeed, when glucose was the substrate, mitochondrial respiration rates were lower in neuronal and astrocyte primary cultures from BAD knockout mice and BAD Ser155A mutant mice compared to wild-type controls. The opposite occurred when β -hydroxybutyrate (5 mM) was the substrate, i.e., respiration rates were higher in neuronal and astrocyte cultures from BAD mutants relative to wild-type mice. Also, seizure severity during kainate- or pentylenetetrazole-induced status epilepticus was reduced in BAD knockout mice and BAD Ser155A mutant mice compared to wild-type controls. The authors inferred that metabolic alterations occurred *in vivo* with glucose and ketone metabolism, decreasing and increasing, respectively, in the BAD mutant mice, and were hence responsible for the increased seizure thresholds. However, this was not directly tested (Giménez-Cassina et al., 2012). While intriguing, this study failed to demonstrate directly the relevance of BAD Ser155 to the anti-seizure or neuroprotective effects of a KD or β -hydroxybutyrate. Thus, how BAD relates specifically to β -hydroxybutyrate-evoked anti-seizure mechanisms remains unclear.

4.2. Neurotransmitter systems

In general, studies have failed to demonstrate a direct action of ketone bodies on GABA or glutamate receptors at physiologically relevant concentrations (Thio et al., 2000; Yang et al., 2007). Alternatively, ketone bodies may induce homeostatic effects on these neurotransmitter systems, but whether this occurs, and in which direction, are debated questions. Ketone bodies have been shown to increase GABA levels in rat synaptosomes prepared from the forebrains of rodents injected with β -hydroxybutyrate and in cultured astrocytes (Erecinska et al., 1996; Daikhin and Yudkoff, 1998; Suzuki et al., 2009). Supporting these pre-clinical data, patients on a KD showed increased GABA levels in the cerebrospinal fluid, consistent with what was reported using magnetic resonance spectroscopy (Wang et al., 2003; Dahlin et al., 2005). However, more often than not, *in vivo* and *in vitro* studies have reported that ketone bodies do not change total GABA or glutamate content, but rather the preferential source of amino acid carbons shifts from glucose to ketone bodies (Yudkoff et al., 2001; Lund et al., 2009, 2011; Valente-Silva et al., 2015; Zhang et al., 2015). Yet, a consistent finding in these studies is that ketone body metabolism

decreases aspartate. Aspartate is a known inhibitor of glutamate decarboxylase, an enzyme involved in the generation of GABA from glutamate. Therefore, the GABA hypothesis remains viable, as a decrease in aspartate could theoretically promote the synthesis of GABA (McNally and Hartman, 2012).

Another method to regulate neurotransmission is to alter synaptic vesicle loading. Through a series of elegant experiments, Juge et al. (2010) demonstrated that acetoacetate inhibits SLC17 vesicular neurotransmitter transporters. In this family of transporters are vesicular glutamate transporters (VGLUTs) which are responsible for filling presynaptic vesicles with glutamate in a Cl^- -dependent manner. Reconstituting purified VGLUTs in proteoliposomes, these investigators demonstrated that acetoacetate competitively inhibits the allosteric activation by Cl^- of VGLUTs. Significant inhibition occurred in the low μM range, and near complete inhibition was achieved in the low mM range (concentrations that are physiologically relevant). Impressively, Juge and colleagues showed that acetoacetate reversibly inhibited glutamate release from both cultured rat neurons and mouse CA1 pyramidal neurons in hippocampal slices, observations that correlated with decreased miniature excitatory postsynaptic current EPSC amplitude and frequency. Importantly, acetoacetate failed to affect VGATs (vesicular GABA transporters) and miniature IPSCs. Finally, acetoacetate was able to suppress *in vivo* glutamate release and seizures in rat brains exposed to 4-AP (Juge et al., 2010). If there is any criticism of this intricate and challenging study, it is that only the 10 mM acetoacetate concentration was used in the cultures, slices and *in vivo* experiments, leaving open the question of what effects a ~50% inhibition by μM concentrations of acetoacetate or β -hydroxybutyrate would have on synaptic neurotransmission and seizure activity. Finally, acetoacetate was found to be as efficacious and potent an inhibitor of other SLC17 transporters, VNUT (vesicular nucleotide transporter) and VEAT (vesicular excitatory amino acid transporter). In the case of VNUT, which loads ATP into vesicles, this action seems paradoxical because it would lower synaptic inhibition by ATP and adenosine release. However, this loss of inhibition may be outweighed by the reduction of released glutamate.

4.3. ATP-sensitive potassium (K_{ATP}) channels

Potassium channels are a heterogeneous group of ion channels that largely function to hyperpolarize the cell membrane. Inwardly rectifying ATP-dependent potassium (K_{ATP}) channels are inhibited by ATP and open when ATP levels are low. Thus, they represent a direct link between membrane excitability and the metabolic state of the cell. Although there are clear data demonstrating that acetoacetate and β -hydroxybutyrate (0.5–1 mM) increase intracellular ATP levels (Kim et al., 2010), experiments in substantia nigra pars reticulata GABAergic neurons and hippocampal dentate granule cells revealed that K_{ATP} channels are activated indirectly in the presence of acetoacetate (2 mM) or β -hydroxybutyrate (2 mM), thereby decreasing neuronal excitability (Ma et al., 2007; Tanner et al., 2011). Opening of K_{ATP} channels resulted in decreased spontaneous firing rates, and was prevented by the K_{ATP} channel inhibitor, tolbutamide. Further, the ketone body effect was abrogated in Kir6.2 knockout mice (lacking the K_{ATP} channel alpha subunit).

Interestingly, the neuroprotective effects of ketone bodies (cocktail of 1 mM acetoacetate and 1 mM β -hydroxybutyrate) during oxidative stress were shown to involve both surface K_{ATP} channels and mitochondrial K_{ATP} channels (Kim et al., 2015a). Also, 1 mM β -hydroxybutyrate-induced opening of K_{ATP} channels was involved in neurotransmission of cultured cerebellar neurons, as addition of glibenclamide, another K_{ATP} channel inhibitor,

increased D-[2,3-³H]-aspartate release (Lund et al., 2015). Thus far, the most reasonable explanation for the collective and discrepant findings has been proposed by Kawamura et al. (2010). Conducting patch-clamp experiments on hippocampal CA3 pyramidal cells, they found that under conditions of low intracellular glucose and elevated ATP, the large pannexin-1 hemichannels open and release ATP. ATP is then broken down to adenosine by ectonucleotidases in the extracellular space. The increase in extracellular adenosine was shown to activate G-protein coupled A1 receptors which were then linked to the opening of K_{ATP} channels via second messenger signaling (Kawamura et al., 2010). Thus, β-hydroxybutyrate-induced increased ATP production has been proposed as a form of metabolic autocrine regulation that ultimately reduces neuronal and network excitability.

4.4. Histone (lysine) deacetylases and gene regulation

Histone deacetylases (HDACs) are enzymes that remove acetyl groups from lysine residues on histones and other proteins such as transcription factors and other enzymes. Deacetylation of histones loosens the tight wrapping of DNA, thus enabling gene transcription, whereas deacetylation of transcription factors or enzymes can increase or decrease their activity. Shimazu et al. (2013) found that β-hydroxybutyrate inhibits HDAC1, HDAC3 and HDAC4 with IC₅₀s of 5.3, 2.4 and 5.4 mM, respectively – suggesting the physiological relevance of this mechanistic action. Furthermore, they showed that β-hydroxybutyrate-induced HDAC inhibition resulted in up-regulation of genes in the FOXO3A transcription factor network, including the anti-oxidants catalase, mitochondrial superoxide dismutase (SOD2) and metallothionein 2, collectively leading to reduced oxidative stress in kidney cells (Shimazu et al., 2013). Supporting this downstream effect on anti-oxidants, co-application of acetoacetate (1 mM) and β-hydroxybutyrate (1 mM) were earlier found to increase catalase activity in hippocampal slices during oxidative stress (Kim et al., 2010). HDAC3 is also part of the co-repressor complex that inhibits peroxisome proliferator-activated receptor (PPAR) γ, a transcription factor that also regulates many of the same antioxidant genes. Therefore, an HDAC inhibitor would be expected to increase the transcriptional activity of PPARγ (Ye, 2013). In cultured HT22 cells, 5 mM acetoacetate increased PPARγ expression and in *in vivo* experiments, genetic or pharmacologic loss of PPARγ attenuated the anti-seizure effects of the KD (Jeong et al., 2011; Simeone et al., 2017).

An alternative and complementary action could be that ketone bodies increase the expression and activity of the NAD⁺-dependent histone deacetylase sirtuin 3 (SIRT3). In a mouse model of ischemic stroke, administration of ketone bodies reduced the infarct size and oxidative stress and increased protein expression of mitochondrial SIRT3, FOXO3A and SOD2 (Yin et al., 2015). In primary neuronal cultures, ketone bodies (cocktail of 0.4 mM β-hydroxybutyrate and 0.45 mM acetoacetate) prevented cell death from rotenone-induced mitochondrial dysfunction and increased mitochondrial content of SIRT3, FOXO3A and Mn-SOD. Importantly, ketone body neuroprotection was lost when SIRT3 shRNA, and FOXO3A and SOD2 expression were inhibited (Yin et al., 2015).

4.5. Anti-inflammatory effects

Experimental and clinical research within the last two decades has provided convincing evidence of the importance of inflammatory mediators and related molecules in ictogenesis, epileptogenesis, and the exacerbation of seizure severity. Importantly, seizures induce inflammation and increase levels of cytokines and chemokines such as interleukin-1β (IL-1β) (Vezzani and Granata, 2005; Vezzani et al., 2011). These factors are secreted from both

activated glia and neurons in many experimental models of acute and chronic seizures and increase neuronal and network hyperexcitability. Inflammatory mediators also increase the permeability of the blood-brain barrier allowing the infiltration of monocytes and macrophages into the brain, which further enhances the inflammatory process (Marchi et al., 2011, 2014). Recently, β-hydroxybutyrate has been found to modulate two inflammatory mechanisms.

HCA2: Hydroxy-carboxylic acid receptor 2 (HCA2, GPR109A) is a Gi protein-coupled receptor that is activated by β-hydroxybutyrate. Rahman et al. (2014) sought to determine whether HCA2 contributes to the neuroprotective effects of the KD and β-hydroxybutyrate. They induced ischemic strokes by occluding the distal middle cerebral artery. Mice fed a KD or administered β-hydroxybutyrate via subcutaneous minipumps achieved plasma concentrations of 1 mM and developed smaller infarcts than control mice. This protection by the KD and β-hydroxybutyrate was lost in HCA2^{-/-} mice. Furthermore, using cell ablation techniques and chimeric mice, these investigators demonstrated that HCA2 is expressed in infiltrating bone marrow-derived monocytes and macrophages in the brain, and that HCA2 activation on these monocytes and macrophages is required for the neuroprotective effect (Rahman et al., 2014).

NLRP3: The innate immune sensor NOD-like receptor protein 3 (NLRP3) inflammasome is a multi-protein complex that controls the activation of caspase-1 and the release of the pro-inflammatory cytokines IL-1β and IL-18 in macrophages. In response to diverse damage-associated molecular patterns such as excess glucose ceramides and amyloids, the macrophage experiences a loss of cytoplasmic K⁺, promoting oligomerization of apoptosis-associated speck-like protein containing a caspase activation and recruitment domain or ASC, speck formation, and the assembly of the inflammasome (Youm et al., 2015). In an elegant study, Youm and colleagues found that β-hydroxybutyrate (1–10 mM), but not acetoacetate (10 mM), inhibits NLRP3 inflammasome assembly by preventing K⁺ efflux. Although the exact mechanism(s) of β-hydroxybutyrate inhibition remain unclear, this effect on NLRP3 assembly was not dependent on chirality or starvation-regulated mechanisms like AMP-activated protein kinase (AMPK), ROS, autophagy or glycolytic inhibition. β-hydroxybutyrate inhibition was also independent of uncoupling protein-2, SIRT2 and HCA2 (Youm et al., 2015). Interestingly, another study suggested that *in vivo* and *in vitro* inhibition of the inflammasome by 1 mM β-hydroxybutyrate occurs through suppression of endoplasmic reticulum-related oxidative stress (Bae et al., 2016). Additionally, β-hydroxybutyrate increased SOD2 and catalase by mediating FOXO3 through AMPK activation, implying that decreasing ROS may have prevented inflammasome activation. Nevertheless, β-hydroxybutyrate reduced NLRP3 inflammasome-mediated IL-1β and IL-18 production in human monocytes. Finally, in multiple mouse models of NLRP3-mediated diseases in which mutant NLRP3 is constitutively active, *in vivo* administration of β-hydroxybutyrate or a KD attenuated caspase-1 activation and IL-1β secretion (Youm et al., 2015).

5. Summary

Within only the past few years, there has been remarkable progress in our understanding of ketone bodies and their numerous biological effects (Puchalska and Crawford, 2017). Indeed, circling back to the original question of whether ketone bodies exert anti-seizure effects, accumulating evidence suggests that they play a pivotal role and are not just epiphenomena. While some of the novel biological effects observed and targets activated by ketone bodies such as β-hydroxybutyrate have not yet been shown in

brain, it is reasonable to extrapolate these findings as fundamental mechanisms such as HDAC inhibition would not be expected to be restricted to only one tissue type. Clearly, ketone bodies can no longer be considered simply energy molecules or substrates for membrane biosynthesis during development (Morris, 2005; Puchalska and Crawford, 2017). Rather, it is increasingly becoming apparent that ketone bodies contribute to the anti-seizure efficacy of the KD. However, ultimate validation of their role in the clinical setting has yet to be firmly demonstrated.

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