The New Ketone Alphabet Soup: BHB, HCA, and HDAC

Suppression of Oxidative Stress by β-Hydroxybutyrate, an Endogenous Histone Deacetylase Inhibitor.

Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, Grueter CA, Lim H, Saunders LR, Stevens RD, Newgard CB, Farese RV Jr, de Cabo R, Ulrich S, Akassoglou K, Verdin E. *Science* 2013;339(6116):211–214.

Concentrations of acetyl–coenzyme A and nicotinamide adenine dinucleotide (NAD^+) affect histone acetylation and thereby couple cellular metabolic status and transcriptional regulation. We report that the ketone body D-β-hydroxybutyrate (βOHB) is an endogenous and specific inhibitor of class I histone deacetylases (HDACs). Administration of exogenous βOHB, or fasting or calorie restriction, two conditions associated with increased βOHB abundance, all increased global histone acetylation in mouse tissues. Inhibition of HDAC by βOHB was correlated with global changes in transcription, including that of the genes encoding oxidative stress resistance factors FOXO3A and MT2. Treatment of cells with βOHB increased histone acetylation at the *Foxo3a* and *Mt2* promoters, and both genes were activated by selective depletion of HDAC1 and HDAC2. Consistent with increased FOXO3A and MT2 activity, treatment of mice with βOHB conferred substantial protection against oxidative stress.

The β-Hydroxybutyrate Receptor HCA2 Activates a Neuroprotective Subset of Macrophages.

Rahman M, Muhammad S, Khan MA, Chen H, Ridder DA, Müller-Fielitz H, Pokorná B, Vollbrandt T, Stölting I, Nadrowitz R, Okun JG, Offermanns S, Schwaninger M. *Nat Commun* 2014;5:3944. doi:10.1038/ncomms4944.

The ketone body β-hydroxybutyrate (BHB) is an endogenous factor protecting against stroke and neurodegenerative diseases, but its mode of action is unclear. Here we show in a stroke model that the hydroxy-carboxylic acid receptor 2 (HCA_2, GPR109A) is required for the neuroprotective effect of BHB and a ketogenic diet, as this effect is lost in *Hca2*$^{-/-}$ mice. We further demonstrate that nicotinic acid, a clinically used HCA_2 agonist, reduces infarct size via a HCA_2-mediated mechanism, and that noninflammatory Ly-6C^{Lo} monocytes and/or macrophages infiltrating the ischemic brain also express HCA_2. Using cell ablation and chimeric mice, we demonstrate that HCA_2 on monocytes and/or macrophages is required for the protective effect of nicotinic acid. The activation of HCA_2 induces a neuroprotective phenotype of monocytes and/or macrophages that depends on PGD_2 production by COX1 and the haematopoietic PGD_2 synthase. Our data suggest that HCA_2 activation by dietary or pharmacological means instructs Ly-6C^{Lo} monocytes and/or macrophages to deliver a neuroprotective signal to the brain.

Commentary

After nearly a century of clinical use, the mechanisms underlying the anti-seizure effects of the ketogenic diet (KD) remain unclear. Notwithstanding this dearth of knowledge, the KD's clinical utility appears to be expanding, as there is increasing evidence of its broad neuroprotective properties (1, 2). For both anti-seizure and neuroprotective actions, there have emerged a broad array of proposed mechanisms, and more recently, biochemical and cellular effects that would not necessarily be predicted for bioenergetic substrates and enzymes (3). However, it is uncertain which putative mechanisms are relevant in the clinical context. Clearly, as perturbations in cellular metabolism are increasingly linked to the pathogenesis of neurologic disorders (4), a better mechanistic understanding of the KD (and indeed its variants, such as the modified Atkins diet and the low-glycemic index therapy), would potentially lead to more effective dietary/metabolic treatments, and even perhaps a "KD in a pill," although the evidence thus far suggests that modulation of a single target mechanism is unlikely to recapitulate the entire clinical profile of the KD (5).

Not surprising, some investigators have taken a simple reductionist approach to studying KD mechanisms and asked whether the principal by-products of fatty acid oxidation (i.e., ketone bodies such as β-hydroxybutyrate [BHB], acetoacetate [ACA], and acetone) might exert direct effects on brain network excitability, beyond their well-established role as alternative fuels for bodily tissues under conditions of decreased bioavailability of glucose. Thus far, the preclinical data support the notion that ACA and acetone can exert antiseizure effects when acutely administered in vivo, but whether BHB possesses antiseizure activity remains unknown (3). And unfortunately,

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the clinical data have not been helpful in resolving the issue of whether ketone bodies are important mediators or epiphenomena (6).

Against this backdrop, there have emerged two intriguing studies that have provided new insights into the age-old question of how the KD works. The first relates to ketone-induced epigenetic regulation through histone modifications, which may play a pivotal role in ictogenesis and epileptogenesis (7). Histones are important proteins that regulate chromatic structure in eukaryotic cells and are heavily post translationally modified. H3 is one of five main histone proteins whose sequence variants and variable modification states critically modulate long-term regulation of genes. Acetylation of lysine residues on histones is mediated by acetyltransferases, which enable unbound DNA to undergo transcription, whereas removal of acetyl groups by histone deacetylases (HDACs) results in tight binding of histones to DNA and transcriptional repression. HDAC inhibitors are increasingly becoming recognized as potentially important anticancer and anti-inflammatory agents (8). With respect to epilepsy, the most notable example of an HDAC inhibitor is the broad-spectrum anticonvulsant valproic acid (VPA), which inhibits both class I and II HDACs (9) and is cytotoxic to many different cancer types (8).

Shimazu and colleagues noted that BHB is structurally related to butyrate, a product of anaerobic fermentation in bacteria, and which is known to be a small-molecule inhibitor of class I and class II HDACs. Based on this observation, these investigators asked whether the major ketone body BHB might exhibit similar activity against HDACs. They exposed HEK293 (human embryonic kidney) cells in vitro to BHB for 8 hours and used antibodies against acetylated H3 (both lysine-9 and lysine-14 isoforms) to show that BHB increased histone acetylation in a dose-dependent manner, and importantly, over a clinically relevant concentration range (i.e., 1–2 mM) (6, 10).

To determine the selectivity of this effect, Shimazu and coinvestigators purified recombinant human HDACs, incubated them with labeled acetylated histone peptides, and then measured their deacetylase activity. They found that BHB inhibited HDAC1, HDAC3, and HDAC4 (but not HDAC6) in a dose-dependent manner (IC_{50}s of 2.4–5.3 mM). Of interest, they also found that "supratherapeutic" concentrations of acetoacetate also inhibited class I and class IIa HDACs in vitro and in HEK293 cells. Further evidence was provided by depleting cells of BHB dehydrogenase with a small interfering RNA (siRNA), which suppressed histone acetylation by BHB at concentrations up to 3 mM. Finally, to establish an in vivo effect of BHB, they fasted or calorie restricted mice, or instead implanted osmotic mini-pumps loaded with BHB. All of these approaches led to BHB concentration increases in mouse serum ranging from 0.6 to 1.5 mM. Tissues were collected and analyzed for histone acetylation using immunoblotting, which showed that histone acetylation increased up to several-fold in multiple organs, especially the kidney. Inhibition of HDACs by BHB correlated with global changes in transcription, particularly genes encoding oxidative stress resistance factors such as *Foxo3a* and *Mt2*. Similarly, depletion of class I and II HDACs with short hairpin–mediated RNAs (shRNAs) led to activation of these genes, providing evidence that BHB induces local histone acetylation at the promoter of oxidative stress-resistance genes by inhibiting HDACs 1 and 2. Taken together, these results provide compelling evidence that BHB is an endogenous HDAC inhibitor which confers cellular proection against oxidative stress by regulating genes involved in redox homeostasis.

In a second study by Rahman and colleagues, and relevant to the neuroprotective effects of ketone bodies and KDs (11, 12), a unique target mechanism was recently identified. Adipocytes typically release free fatty acids, but this process is prevented by BHB, acting via the HCA_2 (also known as NIACR1 or niacin receptor 1), a G protein-coupled receptor found on adipocytes, neutrophils, tissue macrophages, and in the anterior cingulate cortex (13, 14). Thus, Rahman and colleagues asked whether the beneficial effects of the KD are mediated via the HCA_2 receptor. These investigators used the middle cerebral artery occlusion model of stroke to study potential neuroprotective effects of various pharmacologic interventions. Pretreatment with a KD for 18 days was associated with a smaller infarct volume in wild-type, but not HCA_2 knock-out, mice. Similar findings were noted when mice (of both genotypes) were pretreated with BHB for 10 hours. The importance of the HCA_2 receptor was further provided by demonstration of tissue protection when nicotinic acid (another ligand of the HCA_2 receptor) was administered 10 minutes prior to stroke induction in wild-type, but not knock-out, mice. In a more clinically relevant model, nicotinic acid also protected against stroke-mediated damage when given after stroke induction, although the magnitude of the effect was somewhat decreased. Of importance, stroke-induced behavioral abnormalities also were decreased in wild-type mice treated with BHB or nicotinic acid. Collectively, these studies indicate that HCA_2 receptors are necessary for ketone body– and nicotinic acid–mediated prevention of tissue destruction after stroke.

Rahman and colleagues sought to determine which cells were responsible for mediating this protective effect. They used a genetically modified reporter mouse in which monomeric red fluorescent protein (mRFP) expression is directed by the *Hca2* locus (*Hca2*^{mRFP}). At baseline, mRFP was noted only in resident $CD11b^+$ microglia, but after stroke induction, mRFP-positive cells were noted in the periphery of the ischemic zone. This observation could be explained by two distinct possibilities: either resident microglia were reactive or there was infiltration of monocytes/macrophages from peripheral blood. The authors implemented a clever strategy to distinguish between these possibilities using chimeric mice, with wild-type bone marrow transplanted to *Hca2*^{mRFP} mice and *Hca2*^{mRFP} marrow transplanted to wild-type mice, as well as flow cytometry of tissue and peripheral blood. This series of experiments demonstrated that both resident microglia were activated, and furthermore, bone marrow–derived monocytes/macrophages infiltrated the ischemic periphery.

The more surprising finding was that in blood, the number of "inflammatory" types of monocytes/macrophages decreased after stroke, which raised the possibility that "inflammatory" monocytes/macrophages differentiated into the "resident" (neuroprotective) subtype in tissue after infiltration. Experiments with different chimeric mice (using bone-marrow transplants of wild-type and *Hca2* knock-out mice) showed that HCA_2 in bone marrow–derived cells were responsible for nicotinic acid–mediated prevention of tissue destruction.

Because other cell types express HCA_2 (including neutrophils, which may play a role in the immunologic response to stroke), the authors used mice expressing the human diphtheria toxin receptor under the control of the monocyte/macrophage-specific CD11b promoter to demonstrate that nicotinic acid–mediated protection against stroke requires the presence of monocytes/macrophages.

One of the major problems with using nicotinic acid clinically is a cutaneous flushing response mediated via prostaglandin D2 (PGD_2) (15), which is synthesized in response to activation of HCA_2 receptors after nicotinic acid binding. PGD was shown previously to have neuroprotective effects in a neonatal hypoxia–ischemia model (16). Consistent with the hypothesis that the protective effect of HCA_2 receptor–mediated neuroprotection is mediated via PGD_2, Rahman and colleagues found that PGD_2 levels are elevated in both plasma and brain after nicotinic acid treatment. Further, the post stroke effect of nicotinic acid was eliminated in mice lacking one of the major enzymes responsible for PGD_2 synthesis in macrophages, COX1. Administration of a small molecule inhibitor of the other major PGD synthetic enzyme (hematopoietic PGD_2 synthase) also partially reversed the neuroprotective effect of nicotinic acid. To summarize, pharmacologic activation of one BHB receptor, HCA_2, led to neuroprotection in a stroke model, and this effect was mediated via PGD synthesis, primarily in cells of the monocyte/macrophage lineage that infiltrate margins of the ischemic zone from peripheral blood.

While the studies by Shimazu and colleagues and Rahman and co-investigators are indeed elegant in design and innovative in scope, there remain a number of unanswered questions. Do either or both of these mechanisms (i.e., modulation of histone acetylation and the HCA_2 receptor) play a role in ictogenesis or epileptogenesis? Neither study provides direct evidence in this regard, but based on emerging lines of research (1, 2, 7), both strongly indicate that BHB (and by extension, the KD) could influence the epileptic state. In support of this, the KD has recently been shown to affect epigenetic changes in epileptic brain (17) and is also proving useful in patients with neuroinflammation-related epilepsy (18). With respect to neuroprotection, there are indeed many other KD-related mechanisms that likely play a role (3), such as ketone-mediated inhibition of vesicular glutamate release (19). And as recognized by Rahman and colleagues, another potential mechanism may be the PGD_2 metabolite (15d-PGJ), an agonist of neuroprotective peroxisome proliferator alpha receptor gamma ($PPAR_\gamma$). Taken together, the recent studies by Shimazu and coinvestigators and Rahman and colleagues have revealed molecular mechanisms that may be relevant for KD-induced neuroprotection, and possibly, epileptogenesis. Although much more work needs to be done, the mystery of how the KD may induce lasting protective changes in the brain is now starting to be unraveled.

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