

# An Ester of $\beta$ -Hydroxybutyrate Regulates Cholesterol Biosynthesis in Rats and a Cholesterol Biomarker in Humans

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**Abstract** In response to carbohydrate deprivation or prolonged fasting the ketone bodies,  $\beta$ -hydroxybutyrate ( $\beta$ HB) and acetoacetate (AcAc), are produced from the incomplete  $\beta$ -oxidation of fatty acids in the liver. Neither  $\beta$ HB nor AcAc are well utilized for synthesis of sterols or fatty acids in human or rat liver. To study the effects of ketones on cholesterol homeostasis a novel  $\beta$ HB ester (KE) ((*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate) was synthesized and given orally to rats and humans as a partial dietary carbohydrate replacement. Rats maintained on a diet containing 30-energy % as KE with a concomitant reduction in carbohydrate had lower plasma cholesterol and mevalonate (−40 and −27 %, respectively) and in the liver had lower levels of the mevalonate precursors acetoacetyl-CoA and HMG-CoA (−33 and −54 %) compared to controls. Whole liver and membrane LDL-R as well as SREBP-2 protein levels were higher (+24, +67, and +91 %, respectively). When formulated into a beverage for human consumption subjects consuming a KE drink (30-energy %) had elevated plasma  $\beta$ HB which correlated with decreased mevalonate, a liver cholesterol synthesis biomarker. Partial replacement of dietary carbohydrate with KE induced ketosis and altered cholesterol homeostasis in rats. In healthy

individuals an elevated plasma  $\beta$ HB correlated with lower plasma mevalonate.

**Keywords** Cholesterol biosynthesis ·  $\beta$ -Hydroxybutyrate · Diabetes · Ketogenic diet · Mass spectrometry · Statins · Liver · Triglycerides · Human · Dietary supplement

## Abbreviations

ACAC	Acetoacetate
$\beta$ HB	$\beta$ -Hydroxybutyrate
CE-MS	Capillary Electrophoresis Mass Spectrometry
KE	Ketone ester
SREBP-2	Sterol response element binding protein 2

## Introduction

Hypercholesterolemia contributes significantly to the development of coronary artery disease and controlling blood LDL-cholesterol through statin therapy has become a routine intervention [1]. Statins inhibit the HMG-CoA reductase (HMGCR) mediated production of mevalonate—the rate determining step in sterol biosynthesis. The lower amount of liver cholesterol signals an increase in hepatic reabsorption of LDL particles [2, 3] thereby decreasing atherosclerotic plasma lipoproteins [4]. The ketone bodies,  $\beta$ -hydroxybutyrate ( $\beta$ HB) and acetoacetate (AcAc), which are produced by the liver in response to carbohydrate deprivation or prolonged fasting are not well utilized by the liver for synthesis of liver sterols or fatty acids [5, 6]. Other laboratories have demonstrated lipid effects of ketone bodies, physiological concentrations of either  $\beta$ HB or AcAc suppresses transcriptional activity of carbohydrate response element binding protein

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(ChREBP), an activator of fatty acid synthesis [7] and there has been one report of ketone supplementation lowering blood cholesterol in humans [8]. Moderating dietary carbohydrate intake with ketones may decrease biosynthesis of liver cholesterol by limiting the availability of sterol biosynthetic precursors, mevalonate, acetoacetyl-CoA and HMG-CoA.

Ketogenic diets (low carbohydrate, low protein and high fat) are typically used to increase blood ketone levels in humans, however, prolonged calorie restriction in general also induces ketosis [9]. Both of these methods have challenges, high fat ketogenic diets are not able to raise blood ketones beyond 1.5 mM and are often not palatable, alternatively, prolonged caloric restriction is more effective at raising blood levels but it presents serious issues with patient compliance. We have developed an alternative method to rapidly elevate blood ketones with minimal dietary modification which avoids the stated issues with these other methods. (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate, a novel ketone ester (KE), is hydrolyzed during digestion, and oxidized in the liver, forming two molecules of  $\beta$ -hydroxybutyrate. The KE has been demonstrated to safely increase blood ketone levels to 5–6 mM in humans and rodents [10, 11]. Pharmacokinetics and toxicology of the ester have been described by Clarke *et al.* [10, 11] and the ketone ester has been found to be safe and well tolerated in humans and rodents. The KE has been used to rescue behavioral and cognitive phenotypes in a transgenic mouse model of Alzheimer's disease, and also diminished the accumulation of phosphorylated Tau and beta-amyloid in the hippocampal regions [12]. Moreover, when given orally to an Alzheimer's patient, the KE was shown to improve a variety of behavioral and cognitive performance outcomes [8]. Interestingly, several elements of the patient's blood lipid panel also improved dramatically when maintained on the KE.

In this study we examined the effects of maintaining rats for 30 days on a 30-energy % liquid KE diet on several parameters of cholesterol homeostasis. We also determined plasma concentrations of  $\beta$ HB and mevalonate, a biomarker of liver cholesterol biosynthesis in three groups of healthy human subjects each of whom consumed a single KE beverage [13].

## Materials and Methods

### Animal Study

The animal study was reviewed and approved by the Animal Care and Use Committee of NIAAA, National Institutes of Health. Male Sprague–Dawley Rats weighing 230–270 g were obtained from Charles River Wilmington, MA. Animals were divided into 2 groups- a pair fed liquid control (Lieber-DeCarli '82 Control Diet, caloric content: 15 % fat, 36 % protein and 49 % carbohydrate) (Bio-Serv, Flemington, NJ) [14] and an *ad libitum* experimental KE liquid diet (composed of a 5:1 ratio of the Lieber-DeCarli '82 Ethanol Diet: Control Diet: having a caloric content of: 15 % fat, 36 % protein, 19 % carbohydrate and 30 % KE). The control group's daily calories were matched to the KE diet group average consumption on the previous day [14, 15]. Animals were fed for 30 days on their respective diets. The KE group was fed initially with 15 % KE diet for 3 days, then 23 % KE diet for 3 days and finally 30 % KE diet for 30 days (a total of 36 days), as previously described [14]. Animals were anesthetized using isoflurane and sacrificed by cardiac puncture and exsanguination. Blood was collected into EDTA vacutainer tubes (BD Biosciences, Franklin Lakes, NJ), plasma was separated by centrifugation (2000G, 10 min, 4 °C). Livers were collected and snap frozen in liquid nitrogen. Tissues were stored at –80 °C until use.

### Human Studies

The healthy human study has been described in Clarke *et al.* [10] and a subset of plasma was obtained from that study for  $\beta$ HB and mevalonate analysis. In short, healthy adult volunteers, both males and females (Table 1), were recruited for a single-center, in patient open-label study that was conducted at DGD Research (later Cetero Research) (San Antonio, Texas, USA). The protocol, including amendments and consent forms, was approved by the Independent Investigational Review Board, the Department of Defense and the University of Oxford Ethics Committees (Protocol Number: CHSI 0701-04). The study was conducted in accordance with the guidelines set forth by the International Conference on Harmonization Guidelines for

**Table 1** Healthy volunteer demographics

Subjects (male/female)	KE mg/Kg	Age	BMI	Glucose (mg/dL)		Cholesterol (mg/dL)	
				Baseline	24 h post	Baseline	24 h post
6 (3/3)	140	35 ± 8	26 ± 2	95 ± 12	87 ± 14	170 ± 34	178 ± 35
6 (3/3)	357	32 ± 9	26 ± 2	99 ± 12	98 ± 9	179 ± 33	183 ± 34
6 (4/2)	714	32 ± 9	25 ± 3	92 ± 8	90 ± 14	173 ± 47	161 ± 48

**Table 2** Macronutrient and caloric content of the KE beverage

Macronutrients	g/100 g	Kcal	% energy
Carbohydrate	6.7	27	32
Protein	3.9	16	19
Fat	1.7	16	19
KE	5.2	25	30
Total	17.5	84	100

Good Clinical Practice, the Code of Federal Regulations for Good Clinical Practice, and the Declaration of Helsinki regarding the treatment of human subjects in a study. Randomization was not performed as a sequential sample design was used (i.e., first enrolled, first tested). Blinding of the medical staff and subjects was not possible since the ketone ester was administered with a supplemental formula (Ensure® Original) as necessary to provide all subjects with equal calories. A single KE beverage (macro nutrient content and amounts specified in Table 2) was consumed in the morning with supplemental Ensure® providing the balance of calories for the Low and Medium KE groups. Additional meals of Ensure® at 4 and 9 h after the KE beverage were consumed providing a total daily caloric consumption of 34 kcal/Kg body weight (16). An indwelling catheter was used to draw blood at baseline, 1, 2, 3, 4, 6, 8, 12 and 24 h after consuming the KE drink.

### Membrane Isolation

Liver plasma membranes were isolated via differential centrifugation using a modified version of the method described in Hubbard AL, Wall DA, and Ma A [16]. Solutions, as described in Hubbard *et al.* [16] and homogenates were kept on ice at all times and centrifugations were performed at 4 °C. Reagents were obtained from Sigma–Aldrich (St. Louis, MO) except Halt™ protease inhibitors which were obtained from Pierce (Rockford, IL). One half gram of frozen liver was first diced and homogenized into 5 ml 0.25 M STM with 10 passes of a loose fitting glass/glass dounce homogenizer. The mixture was centrifuged at 280g for 5 min and supernatant transferred to a new tube. The pellet was resuspended into 3 ml of 0.25 M STM and centrifuged as before and the supernatant was combined with the first. The combined supernatant was centrifuged at 1500g for 10 min, the supernatant was discarded and the pellet was resuspended in 10 ml of 1.42 M STM and transferred into 13.2 ml capacity ultra-clear thin wall centrifuge tubes (Beckman Coulter). A 0.25 M STM solution was layered on top of the 1.42 M STM until nearly filled and the tubes were balanced. Tubes were centrifuged at 82,000g

for 60 min in a swinging bucket (rotor SW-41). The white, fluffy interphase between the layers was collected in high spin centrifuge tubes and TM was added (lowering the sucrose concentration) until the tubes were nearly full and balanced. The tubes centrifuged at 40,000 rpm (270,000g) for 20 min (Type 90 Ti rotor). The pellet was solubilized in 50 µl of 2 % SDS with protease inhibitors, 1 mM DTT, 1 mM NaF, 5 mM EDTA, 10 mM β-glycerophosphate and 250 µM Na<sub>3</sub>VO<sub>4</sub> and sonicated 3 times for 5 s on ice and stored at –80 °C until use.

### Western Blotting

Liver powder was lysed into 2 % SDS lysis buffer (described above) and sonicated 3 times for 5 s on ice, allowed to lyse on ice for 20 min then cleared by centrifugation for 15 min at 12,000g and 4 °C. For both whole liver and membranes, protein concentration was determined using the Biorad DC protein assay, and the lysate was diluted to 3 µg/µl for whole liver and 1 µg/µl for membranes with 4× LDS sample loading buffer (Life Technologies), 10× sample reducing agent (Life Technologies) and ultra-pure water. Each 15 µl sample was loaded into a 4–12 % Bis–Tris NuPAGE Gel (Life Technologies) and run for 30 min at 120 V, then 30 min at 200 V, then transferred onto Immobilon-FL PVDF membrane (Millipore) pre-wetted with methanol, in Tris–Glycine Transfer buffer with 20 % methanol for 1 h at 500 mA. Membranes were blocked for 1 h with Odyssey Block (Licor Biosciences), and then incubated with primary antibody diluted in 5 % milk in Tris buffered saline with 0.1 % Tween-20 (TBST) overnight at 4 °C and then washed 3 × 10 min with TBST before secondary antibody dilutions were applied. The one exception to this was actin, which was diluted as above but only applied to the blot for 1 h at room temperature before washing as above. Secondary antibodies were conjugated with IR-Dye 800 and 680 (rabbit and mouse, respectively, Licor Biosciences). These antibodies were diluted 1:10,000 in 5 % milk in TBST, the secondary antibody solution was incubated on to the membranes for 1 h at room temperature. After secondary antibody incubation, the blots were washed 3 × 10 min with TBST and then 2 × 10 min with TBS without Tween-20. Blots were scanned and quantified using the Odyssey scanning system (Licor Biosciences). Primary antibodies and dilutions: SREBP-2 (1:250, Abcam ab30682), HMG-CoA Reductase (1:500, Abcam ab174830), LDL Receptor (1:500 for whole liver, 1:2000 for membrane preparations, Abcam ab52818), Caveolin-3 (1:5000, Abcam ab2912) and Actin (1:3000, Sigma A5060).

## Real Time PCR

Liver powder (~100 mg) was lysed using 1 ml Trizol reagent (Life Technologies, Carlsbad, CA), isolation of RNA was performed according to the manufacturer's specifications. RNA was dissolved into RNase-free water (Ambion, Austin, TX), diluted to 1 µg/µl and re-precipitated using LiCl (Ambion, Austin, TX) following manufacturer's instructions. All RNA post re-precipitation had 260/280 > 2.05 and 260/230 > 2.1. Re-precipitated RNA (1 µg) was used for reverse transcription using iScript cDNA synthesis kit (Biorad, Hercules, CA). Primers were designed using the NCBI primer design tool and included at least one exon–exon junction. Primer efficiency was also established using dilutions ranging from 5 to 5000 and only primers that exhibited efficiency between 1.95 and 2.05 and did not amplify no-RT controls were used in this study. The primer sequences were as follows; SREBP-2 Forward: CCGAACTGGGCGATGGATG, Reverse: TGTAGCATCTCGTCGATGTCC, LDL Receptor Forward: CACTGAAGCGCAAGGAGGAT, Reverse: CCTTGGACTTGGGAGGACAC, HMG-CoA Reductase Forward: TGAGATCCGGAGGATCCAAG, Reverse: CCA-CAGATCTTGTGTTGCGG and 28S rRNA Forward: CGTACGACTCTTAGCGGTG, Reverse: TGCGTTCGAA-GTGTTCGATGA. Primers were synthesized by Integrated DNA Technologies. RT-PCR was performed using Thermo Fisher Maxima SYBR Green and carried out on an Applied Biosystems 7500 Real Time PCR instrument with an annealing temperature of 60 °C and a synthesis temperature of 72 °C.

## Analytical Procedures

Cholesterol, lanosterol and mevalonate were determined in plasma and liver samples by gas chromatography–mass spectrometry (GC–MS) using the appropriately labeled internal standard for their quantification. The acyl-CoA compounds were measured using capillary electrophoresis–mass spectrometry (CE–MS) using the isotopically labeled acyl CoA standards for their quantification [15]. The number of samples analyzed for each metabolite varied from 4 to 6. The results are presented as means ± SEM.

## Isotopically-labeled Internal Standards and Reagents

Cholesterol-d7 (25, 26, 26, 26, 27, 27, 27) was purchased from Cambridge Isotope Labs (product # DLM-3057-PK Cambridge, MA) and <sup>13</sup>C-mevalonolactone (1, 2) was obtained from Sigma–Aldrich (St. Louis, MO). Lanosterol-d6 product number 700090P was obtained from Avanti Polar Lipids (Alabaster, AL). The <sup>13</sup>C-labeled CoA compounds, malonyl-CoA, acetyl-CoA, and acetoacetyl-CoA

were obtained from Sigma Chemical Co. (St. Louis, MO). The *N*-methyl-*N*-(*tert*-butylmethylsilyl)trifluoroacetamide (MTBSTFA) with 1 % *tert*-butyldimethylchlorosilane (TBD-MCS) reagent was purchased from Pierce (Rockford, IL).

## Perchloric Acid (PCA) Extraction of Liver Samples-Mevalonate Determinations

Liver sample powders (approximately 50 mg) were prepared by perchloric acid extraction as previously described for GC–MS analysis [17]. Samples were extracted into PCA and the <sup>13</sup>C-labeled mevalonate was added in twofold excess of the concentrations of the analyte in liver tissue to the neutralized PCA extracts. The neutralized extracts (50 µl) were then evaporated under a stream of nitrogen to dryness. Samples were immediately reacted with 5 µl of the MTBSTFA + TBDMCS reagent in 15 µl of acetonitrile in 1.5-ml screw-capped glass vials and were heated to 60 °C for 5 min.

## Chloroform–methanol Extraction of Liver Samples for Cholesterol, Lanosterol and Acyl-CoA Determinations

Liver sample powders were extracted using a modified chloroform–methanol extraction procedure [15]. Approximately 50 mg of freeze-clamped liver tissue was added to a frozen (−80 °C) solution of water:chloroform:methanol 300:400:200 µl containing the labeled cholesterol internal standard in amounts commensurate with the concentration of the analyte in tissue (approximately in threefold excess) in 2 ml polypropylene screw capped tubes with 40–50 small glass beads. Samples were shaken on a Mini Bead Beater (Biopsec Products, Bartlesville, OK) for 30 s and repeated until samples were homogeneous. Samples were centrifuged at 4 °C in a Sorvall benchtop centrifuge at a speed of 14,000 g for 15 min to allow the chloroform and water layers to separate. The water layer was removed and filtered through an Amicon Microcon Ultracel YM-10 filter centrifuge tube (Millipore Corp. Bedford, MA) at 14,000 g. The filtrate (about 300 µl) was taken for CE–MS analysis.

## Plasma Extraction for Cholesterol

First, 10 µl of thawed EDTA rat or human plasma was added to 5 µl of D7-cholesterol (0.814 mM) and extracted with a 3:4:2 water:chloroform:methanol solution for determination of the cholesterol content. Samples were vortexed for 2 min and centrifuged at 4 °C in a Sorvall benchtop centrifuge at a speed of 14,000 g for 15 min to allow the chloroform and water layers to separate. The chloroform layer was removed and 20 µl of the solution was taken and

evaporated under a stream of nitrogen for GC–MS analysis. Cholesterol was quantified directly by comparing the peak area ratio of the internal standard to that of the native analyte.

### Analysis of Plasma and Tissue for Mevalonate, Lanosterol and Cholesterol by GC–MS

The specimen extracts were analyzed for mevalonate, lanosterol and cholesterol as their tertiary butyl dimethylsilyl ether derivatives (TBDMS) using gas chromatography–mass spectrometry (GC–MS) in the electron impact mode and quantified using the isotopically-labeled internal standards for either analyte. Aliquots of samples were immediately reacted with 5  $\mu$ l of the MTBSTFA + TBDMCS reagent in 15  $\mu$ l of acetonitrile in 1.5-ml screw-capped glass vials and were heated to 60 °C for 5 min. 1  $\mu$ l of the sample solutions were immediately injected onto the GC–MS capillary column for analysis. Samples were analyzed on an Agilent 5973 quadrupole GC–MS (Agilent, Wilmington, DE) according to previously described methods [15]. The ratio of peak area counts of the labeled internal standard to that of the analyte was used to quantify their concentrations.

### CE-MS Analysis of the Liver Acyl-CoAs

Sample extracts were analyzed on an Agilent capillary electrophoresis-ion trap mass spectrometer (Ultra) using an Agilent 1100 series binary pump to deliver a make-up flow of 50 mM ammonium acetate in methanol:water (50:50) to the electrospray ionization tip (Agilent, Wilmington, DE) according to previously established procedures [15]. The acyl-CoAs were quantified using the ratio of peak area counts of the  $^{13}\text{C}$ -labeled internal standard pseudo molecular ion to that of the analyte.

### Other Assays

Rat insulin levels were measured by ELISA (Alpco, Salem, NH) in plasma according to the manufacturer's specifications. HMG-CoA reductase activity inhibition by ketone bodies was determined using a commercially available kit from Sigma–Aldrich (St. Louis, MO).

### Statistical Analysis

Analysis was performed using GraphPad Prism 6 software. Comparisons of rat plasma and liver measurements (Fig. 1) were performed using Student's *t* test.  $p < 0.05$  was the threshold of significance for all statistical analyses. Statistical comparison for the plasma mevalonate time course (Fig. 3) was done using repeated measures 1-way ANOVA

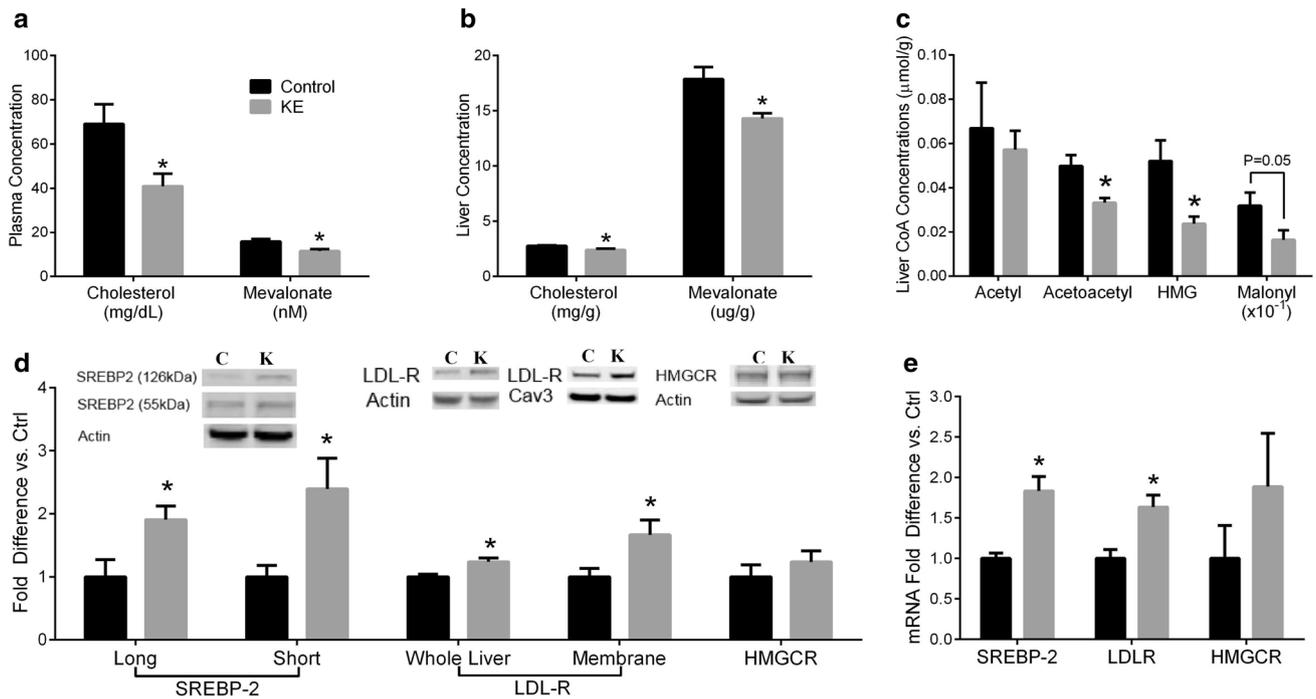
with a Bonferonni *post hoc* test. Correlation between  $\beta$ -hydroxybutyrate and mevalonate was determined by calculating a Pearson product-moment correlation coefficient using all data from all treatment groups and time points, statistical significance was determined by *t* test. Data is presented in text as mean  $\pm$  SEM.

## Results

Rats maintained on the KE diet for 36 days had decreased liver mevalonate precursors, mevalonate and cholesterol, together with increased synthesis of LDL-R compared to pair-fed controls (Fig. 1). KE-fed animals had 40 % lower total plasma cholesterol ( $69.1 \pm 8.9$  vs  $41.0 \pm 5.6$  mg/dl) and 27 % lower plasma mevalonate ( $15.9 \pm 1.1$  vs  $11.5 \pm 0.9$  nM) compared to control animals (Fig. 1a). Livers from KE-fed animals also had 13 % lower total cholesterol ( $2.75 \pm 0.05$  vs  $2.39 \pm 0.13$  mg/g) and 17 % lower mevalonate ( $3.97 \pm 0.21$  vs  $3.29 \pm 0.18$  nmol/g) than pair-fed controls (Fig. 1b).

The two immediate precursors to mevalonate, acetoacetyl-CoA and HMG-CoA, were synthesized from acetyl-CoA, as described in Fig. 2. Concentrations of acetoacetyl-CoA in livers of KE fed animals were 33 % lower ( $0.050 \pm 0.005$  vs  $0.033 \pm 0.002$   $\mu$ mol/g) and levels of HMG-CoA were 54 % lower ( $0.052 \pm 0.009$  vs  $0.023 \pm 0.003$   $\mu$ mol/g) relative to controls (Fig. 1c). In contrast, acetyl-CoA, the precursor of both acetoacetyl- and HMG-CoA, was not significantly altered by the KE diet ( $0.067 \pm 0.021$  vs  $0.057 \pm 0.009$   $\mu$ mol/g). As mevalonate is used in a number of non-cholesterol synthetic pathways, such as ubiquinone synthesis and the prenylation of proteins, the ketone ester's effect on lanosterol was measured in liver samples. Levels of lanosterol in KE fed animals were significantly lower than in controls ( $29.37 \pm 2.68$  vs  $18.27 \pm 3.54$   $\mu$ g/g,  $n = 6$ ,  $p < 0.05$ ) further substantiating the effect of KE diet on steroid biosynthesis. The effect of the KE diet on liver fatty acid synthesis was also of interest and the concentration of the dedicated fatty acid synthetic precursor, malonyl-CoA, which shares the same precursor pool as acetoacetyl-CoA and HMG-CoA, was 50 % lower ( $0.0032 \pm 0.0005$  vs  $0.0016 \pm 0.0005$   $\mu$ mol/g) than controls (Fig. 1c).

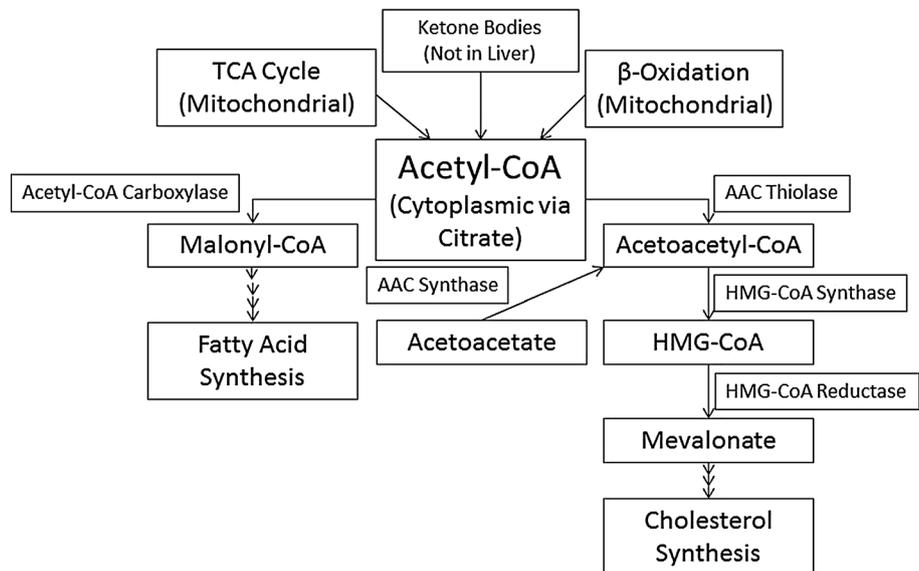
Decreased synthesis of liver cholesterol and lower liver cholesterol levels increase the reabsorption of LDL through the low density lipoprotein receptor (LDL-R) [18]. Long-term KE feeding in rats lowered cholesterol synthesis and increased LDL-R/Cav3 ratios by  $67 \pm 26$  % compared to pair fed controls (Fig. 1d). Furthermore, total LDL-R levels were also increased: LDL-R/actin ratios were  $24 \pm 7$  % higher in the KE fed animals, indicating a whole liver increase in LDL-R protein.



**Fig. 1** Prolonged KE dietary intervention suppressed liver cholesterol synthesis and altered cholesterol homeostasis in rats. **a** Plasma levels of cholesterol and mevalonate were measured by GC–MS. **b** Liver cholesterol and mevalonate were measured by GC–MS. **c** Levels of liver mevalonate CoA precursors (acetyl, acetoacetyl and HMG) as well as the fatty acid precursor malonyl-CoA were measured by isotope dilution CE-MS. **d** Levels of LDL receptor were measured in membrane preparations and in whole liver by Western blot (representative blot presented in *inset*), membrane blot quan-

tification used caveolin 3 as a loading control and whole liver blot quantification used  $\beta$ -actin. SREBP-2 and HMG-CoA reductase (HMGCR) were measured in whole liver samples by Western blot (representative blots presented in *inset*) using  $\beta$ -actin as a loading control. **e** mRNA levels of SREBP-2, LDL-R and HMG-CoA reductase (HMGCR) were measured using RT-PCR and 28 s rRNA as an internal standard.  $N = 4-5$ . \*indicates  $p < 0.05$  by Student's  $t$  test comparing KE fed animals to pair-fed control animals.

**Fig. 2** Diagram for the synthesis and utilization of acyl-CoA precursors in the production of cholesterol and fatty acids. The CoA pools and enzymes listed are located in the cytosol unless otherwise noted



The transcription factor SREBP-2, which controls LDL-R synthesis, has a full length inactive form and a cleaved, transcriptionally active, n-terminal fragment [19].

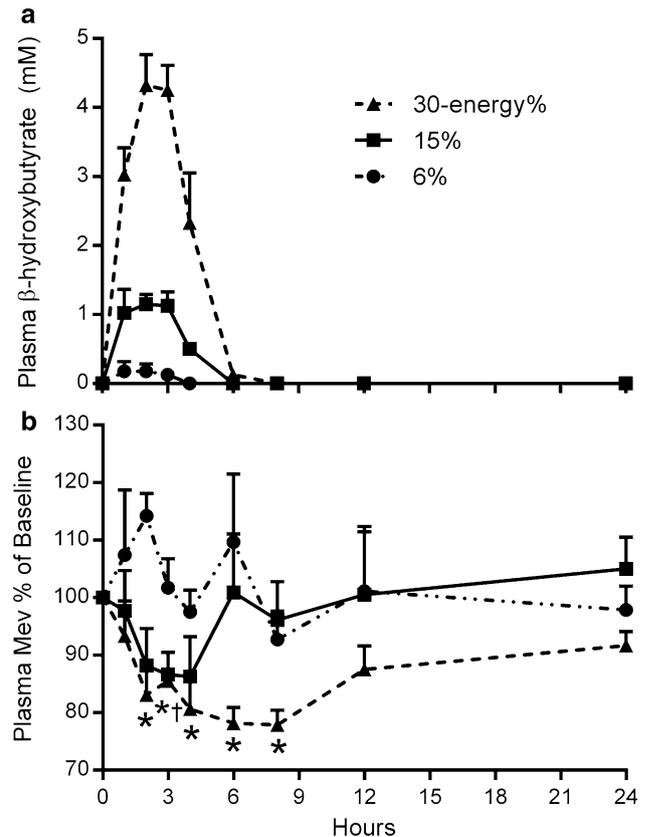
Protein levels of both the full length and cleaved forms of SREBP-2 were increased in the livers of KE fed animals (Fig. 1d). KE -fed animals had full length (126 kDa)

SREBP-2/actin ratios that were  $91 \pm 34 \%$  and truncated (55 kDa) SREBP-2/actin ratios that were  $140 \pm 56 \%$  higher compared to controls. Protein levels of HMG-CoA reductase were not significantly different in the KE fed animals (Fig. 1d).

Cleaved SREBP-2 protein increases transcription of a number of genes that increase liver cholesterol, such as enzymes of the sterol biosynthesis pathway (such as squalene epoxidase, HMG-CoA reductase and squalene synthase) and the LDL-R. Chronically KE fed animals had significantly increased amounts of mRNA of SREBP-2 target genes (Fig. 1e); LDL-R increased by  $63 \pm 18 \%$  and SREBP-2 increased by  $83 \pm 18 \%$ .

Healthy volunteers who consumed a single KE beverage of either 6-energy % (Low KE), 15-energy % (Medium KE) or 30-energy % (High KE) in the morning as a meal replacement had plasma  $\beta$ HB concentrations that increased in proportion to the amount of KE consumed, similar to previously reported findings [10]. Maximum plasma  $\beta$ HB concentrations occurred between 2 and 3 h after consuming the beverage and were sustained for up to 5 h (Fig. 3a). The average maximum plasma  $\beta$ HB concentrations was  $4.3 \pm 0.5$  mM for subjects who consumed the High KE beverage and  $1.15 \pm 0.14$  mM for subjects who consumed the Medium KE amount.

Plasma mevalonate concentrations are tightly correlated with the level of liver cholesterol biosynthesis and statin intake has been shown to acutely decrease plasma mevalonate in humans [13]. Figure 3b shows the baseline normalized plasma mevalonate levels for each of the three groups consuming the KE beverages. As the concentration of plasma mevalonate has been shown to vary throughout the day several blood samples were collected throughout a 24 h period and mevalonate concentrations were compared to their baseline values [13]. The group consuming the Low KE beverage showed no significant changes in plasma mevalonate from baseline concentrations at any time point. Volunteers consuming the Medium KE beverage had significantly decreased ( $-13 \%$ ) plasma mevalonate 3 h after consuming the drink. Subjects consuming the High KE beverage had prolonged suppression of plasma mevalonate levels. Following consumption of High KE beverage plasma mevalonate decreased by 17, 16.5, 19, 22 and 22 % of baseline values at 2, 3, 4, 6 and 8 h, respectively indicating depression of liver cholesterol biosynthesis. The Pearson's correlation coefficient was calculated to determine the overall correlation between plasma levels of  $\beta$ HB and mevalonate using data from all KE dose groups, which established a statistically significant negative correlation ( $r = -0.30$ ,  $n = 108$ ,  $p < 0.01$ ) between the measurements.



**Fig. 3** Time course determinations of plasma  $\beta$ HB and mevalonate suppression in subjects who consumed a KE shake. **a** Total plasma  $\beta$ HB levels were measured at baseline and at a series of time points following consumption of 30, 15 or 6 energy % KE in Ensure<sup>®</sup>. **b** Individual plasma mevalonate measurements were normalized to each subject's baseline measurement; fold change from baseline was calculated and averages were plotted. Repeated measures 1 way ANOVA was used to compare non-normalized mevalonate measurements to respective baseline measurements with a Bonferroni multiple comparisons *post hoc* test, \*indicates  $p < 0.05$  for high dose significantly different from baseline, †indicates  $p < 0.05$  medium dose significantly different from baseline. Pearson correlation coefficient was calculated using all  $\beta$ HB-mevalonate data pairs which established a statistically significant negative correlation between these measurements ( $r = -0.30$ , 108 total pairs,  $p < 0.01$ ). Normalized means  $\pm$  SEM are graphed.  $N = 6$

## Discussion

Elevated blood cholesterol and triglycerides are important risk factors for coronary artery disease [20]. Statins have been highly successful for lowering blood cholesterol, and are currently being prescribed to over 30 million Americans. We demonstrated, using a dietary modification employing a novel KE, a decrease in the availability of cholesterol precursors in the livers of laboratory rats. As hepatocytes from humans and rats have very low succinyl-CoA:3-ketoacid CoA transferase (SCOT) activity

[5, 6], the liver is not able to fully metabolize  $\beta$ HB and AcAc to acetyl-CoA for incorporation into sterols (Fig. 2). We hypothesized that substituting the KE for a portion of carbohydrate calories in the diet would reduce the amount of available acetyl-CoA for synthesis of steroids and fats in the liver. Acetoacetyl-CoA synthase (AACS) can also facilitate incorporation of ketones into lipogenesis [21] as indicated in Fig. 2, but this process is strongly controlled by insulin and feeding [22, 23]. Our laboratory has found that insulin is consistently 50 % lower in KE fed animals [14, 15], which would be expected to significantly decrease AACS activity [22]. Although, Endemann *et al.* [24] and others [25] demonstrated that  $^{14}\text{C}$  labeled  $-\beta$ HB and  $-\text{AcAc}$  can be incorporated into fatty acids and cholesterol in isolated rat liver, we found that when rats were maintained on a diet containing 30 % calories as KE, there were decreased amounts of both liver and plasma cholesterol, as well as liver sterol precursors (mevalonate, lanosterol and CoAs) and malonyl-CoA (Fig. 1a, b). By contrast the feeding of a ketogenic diet increased several measures of plasma cholesterol in human subjects [26].

The origins of acetyl-CoA in the cell are various and may be derived from the decarboxylation of pyruvate from glycolysis, and the oxidation of fatty acids and ketones and also the action of citrate lyase, whereas, the pathways toward anabolic synthesis of fatty acids and steroids from their precursors in the liver is restricted. Fatty acid synthesis is regulated by the energy demands of the system mediated via signaling cascades such as AMP kinase and steroid biosynthesis is complex under the regulation of SREBP-2 but also influenced hormonally by insulin levels [27, 28]. That the levels of acetyl-CoA were unaffected in the ketone ester fed animals indicate that systems leading to its formation and related to its homeostasis were largely unaffected by the composition of the diet.

A direct inhibition of HMGCR by the ketone ester could potentially explain the dose dependent mevalonate suppression observed after consuming a single KE-containing meal in humans (Fig. 3b). However, neither lithium acetoacetate nor sodium  $\beta$ -hydroxybutyrate was found to directly inhibit HMGCR activity *in vitro* when used in concentrations up to 10 mM (data not shown).

Decreased cholesterol synthesis lowers blood cholesterol through increased hepatic reabsorption of cholesterol via enhanced synthesis and membrane localization of the LDL receptor (LDL-R) [18]. This process of increasing LDL-R synthesis is mediated most prominently by the SREBP family of transcription factors, including SREBP-1a, 1c and 2, of which 2 is the most relevant for liver cholesterol homeostasis [27]. As mentioned above, SREBP-2 undergoes proteolytic cleavage in response to low liver cholesterol, ultimately liberating the soluble N-terminus region that initiates gene transcription [19]. We demonstrated that rats maintained for 30 days on the

KE diet had significant increases in SREBP-2 protein, both in its full length and transcriptionally active form (Fig. 1d). Prolonged feeding of the KE diet also increased mRNA of LDL-R, a downstream target of SREBP-2. No significant effects on HMGCR were observed on the transcript or protein levels (Fig. 1e), which seems counter intuitive given the increase in transcriptionally active SREBP-2 and increased LDL-R transcription, but as HMGCR is under significant insulin control, the previously stated insulin decrease associated with KE feeding may also explain this apparent discrepancy.

We extended these findings from this animal study to investigations in humans consuming a single beverage made from the KE. The KE has been shown to be safe for human consumption and is highly effective in elevating blood ketones when consumed in certain quantities [7]. In healthy volunteers, we found that the plasma concentration of  $\beta$ HB varied inversely with that of mevalonate, a biomarker of liver cholesterol synthesis [13]. It appears that a plasma concentration of 1 mM  $\beta$ HB or greater is required to suppress mevalonate concentration (Fig. 3b). Consumption of the High KE beverage was sufficient to maintain prolonged plasma ketone levels as well as depress plasma mevalonate concentration for several hours. Moreover, both  $\beta$ HB and AcAc suppressed the transcription of fatty acid synthetic enzymes in the liver through their effects on ChREBP [7] which may explain in part the decrease in malonyl-CoA levels observed here.

In summary, this work demonstrates for the first time that a dietary modification using a ketone ester can control liver cholesterol biosynthesis by means of limiting the availability of sterol synthetic precursors, acetoacetyl- and HMG-CoA. In rats decreased liver cholesterol lead to lower plasma cholesterol possibly through an upregulated expression of whole liver and membrane LDL-R. In humans a single High KE beverage increased plasma ketones and decreased a liver cholesterol synthesis biomarker within hours of ingestion. Since the periodic elevation of blood ketones through the regular ingestion of the KE has been shown to be therapeutic in an Alzheimer's patient [8] and to decrease neuronal pathology in a transgenic mouse model of the disease [12] a KE-modified diet may be superior to either maintaining a rigorously controlled ketogenic diet, or time-limited severe caloric restriction for mildly cognitive impaired individuals or Alzheimer's patients who would benefit from an induced ketotic state. Although, the present study focused on several parameters of ketone metabolism pertaining to steroid biosynthesis, such as, the determination of liver CoAs and mevalonate concentrations and correlations of plasma beta-hydroxybutyrate levels with mevalonate in humans clearly several important parameters await further investigations, future carefully controlled studies will focus on the effect of diet-induced hormonal influences on the steroid and fatty acid synthesis pathways and more fully delineate enzymatic regulation of acetoacetyl-CoA pools involved in steroid biosynthesis.

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### Compliance with Ethical Standards

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### References

- Allen Maycock CA, Muhlestein JB, Horne BD, Carlquist JF, Bair TL, Pearson RR, Li Q, Anderson JL, Intermountain Heart Collaborative S (2002) Statin therapy is associated with reduced mortality across all age groups of individuals with significant coronary disease, including very elderly patients. *J Am Coll Cardiol* 40:1777–1785
- van der Wulp MY, Verkade HJ, Groen AK (2013) Regulation of cholesterol homeostasis. *Mol Cell Endocrinol* 368:1–16
- Luu W, Sharpe LJ, Gelissen IC, Brown AJ (2013) The role of signalling in cellular cholesterol homeostasis. *IUBMB Life* 65:675–684
- Morris PB, Ballantyne CM, Birtcher KK, Dunn SP, Urbina EM (2014) Review of clinical practice guidelines for the management of LDL-related risk. *J Am Coll Cardiol* 64:196–206
- Fukao T, Song XQ, Mitchell GA, Yamaguchi S, Sukegawa K, Orii T, Kondo N (1997) Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases. *Pediatr Res* 42:498–502
- Williamson DH, Bates MW, Page MA, Krebs HA (1971) Activities of enzymes involved in acetoacetate utilization in adult mammalian tissues. *Biochem J* 121:41–47
- Nakagawa T, Ge Q, Pawlosky R, Wynn RM, Veech RL, Uyeda K (2013) Metabolite regulation of nucleocytoplasmic trafficking of carbohydrate response element-binding protein (ChREBP): role of ketone bodies. *J Biol Chem* 288:28358–28367
- Newport MT, VanItallie TB, Kashiwaya Y, King MT, Veech RL (2015) A new way to produce hyperketonemia: use of ketone ester in a case of Alzheimer's disease. *Alzheimers Dement* 11:99–103
- McPherson PA, McEneny J (2012) The biochemistry of ketogenesis and its role in weight management, neurological disease and oxidative stress. *J Physiol Biochem* 68:141–151
- Clarke K, Tchabanenko K, Pawlosky R, Carter E, Todd King M, Musa-Veloso K, Ho M, Roberts A, Robertson J, VanItallie TB, Veech RL (2012) Kinetics, safety and tolerability of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate in healthy adult subjects. *Regul Toxicol Pharmacol* 63:401–408
- Clarke K, Tchabanenko K, Pawlosky R, Carter E, Knight NS, Murray AJ, Cochlin LE, King MT, Wong AW, Roberts A, Robertson J, Veech RL (2012) Oral 28-day and developmental toxicity studies of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. *Regul Toxicol Pharmacol* 63:196–208
- Kashiwaya Y, Bergman C, Lee JH, Wan R, King MT, Mughal MR, Okun E, Clarke K, Mattson MP, Veech RL (2013) A ketone ester diet exhibits anxiolytic and cognition-sparing properties, and lessens amyloid and tau pathologies in a mouse model of Alzheimer's disease. *Neurobiol Aging* 34:1530–1539
- Parker TS, McNamara DJ, Brown CD, Kolb R, Ahrens EH Jr, Alberts AW, Tobert J, Chen J, De Schepper PJ (1984) Plasma mevalonate as a measure of cholesterol synthesis in man. *J Clin Invest* 74:795–804
- Srivastava S, Kashiwaya Y, King MT, Baxa U, Tam J, Niu G, Chen X, Clarke K, Veech RL (2012) Mitochondrial biogenesis and increased uncoupling protein 1 in brown adipose tissue of mice fed a ketone ester diet. *FASEB J* 26:2351–2362
- Kashiwaya Y, Pawlosky R, Markis W, King MT, Bergman C, Srivastava S, Murray A, Clarke K, Veech RL (2010) A ketone ester diet increases brain malonyl-CoA and uncoupling proteins 4 and 5 while decreasing food intake in the normal Wistar Rat. *J Biol Chem* 285:25950–25956
- Hubbard AL, Wall DA, Ma A (1983) Isolation of rat hepatocyte plasma membranes. I. Presence of the three major domains. *J Cell Biol* 96:217–229
- Srivastava S, Kashiwaya Y, Chen X, Geiger JD, Pawlosky R, Veech RL (2012) Microwave irradiation decreases ATP, increases free [Mg<sup>2+</sup>], and alters *in vivo* intracellular reactions in rat brain. *J Neurochem* 123:668–675
- Charlton-Menys V, Durrington PN (2008) Human cholesterol metabolism and therapeutic molecules. *Exp Physiol* 93:27–42
- Radhakrishnan A, Goldstein JL, McDonald JG, Brown MS (2008) Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. *Cell Metab* 8:512–521
- Krauss RM, Siri PW (2004) Dyslipidemia in type 2 diabetes. *Med Clin North Am* 88:897–909
- Hasegawa S, Noda K, Maeda A, Matsuoka M, Yamasaki M, Fukui T (2012) Acetoacetyl-CoA synthetase, a ketone body-utilizing enzyme, is controlled by SREBP-2 and affects serum cholesterol levels. *Mol Genet Metab* 107:553–560
- Sato H, Takahashi N, Nakamoto M, Ohgami M, Yamazaki M, Fukui T (2002) Effects of streptozotocin-induced diabetes on acetoacetyl-CoA synthetase activity in rats. *Biochem Pharmacol* 63:1851–1855
- Rous S (1977) Fasting and insulin regulation of the utilization of acetoacetate for fatty acid synthesis. *Arch Biochem Biophys* 179:328–333
- Endemann G, Goetz PG, Edmond J, Brunengraber H (1982) Lipogenesis from ketone bodies in the isolated perfused rat liver. Evidence for the cytosolic activation of acetoacetate. *J Biol Chem* 257:3434–3440
- Bergstrom JD, Wong GA, Edwards PA, Edmond J (1984) The regulation of acetoacetyl-CoA synthetase activity by modulators of cholesterol synthesis *in vivo* and the utilization of acetoacetate for cholesterologenesis. *J Biol Chem* 259:14548–14553
- Kwiterovich PO Jr, Vining EP, Pyzik P, Skolasky R Jr, Freeman JM (2003) Effect of a high-fat ketogenic diet on plasma levels of lipids, lipoproteins, and apolipoproteins in children. *JAMA* 290:912–920
- Horton JD, Goldstein JL, Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 109:1125–1131
- Lakshmanan MR, Nepokroeff CM, Ness GC, Dugan RE, Porter JW (1973) Stimulation by insulin of rat liver -hydroxy- -methylglutaryl coenzyme A reductase and cholesterol-synthesizing activities. *Biochem Biophys Res Commun* 50:704–710