The metabolic response to fasting in humans: physiological studies

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5 Muscle *D*-3-hydroxybutyrylcarnitine: an alternative pathway in ketone body metabolism

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Abstract

Background: *D*-3-hydroxybutyrate (*D*-3HB) and acetoacetate play an important role in the adaptation to fasting. It has been suggested by at least two separate studies that *D*-3HB can be coupled to carnitine to form 3-hydroxybutyrylcarnitine. In contrast to *L*-hydroxybutyryl-CoA that can be coupled to carnitine resulting in the formation of *L*-3HB-carnitine, it is unknown whether and how *D*-3HB can be coupled to carnitine resulting in *D*-3HB-carnitine in humans.

Study-aim: To assess which stereo isomers of 3-hydroxybutyrylcarnitine are present in vivo.

Methods: 12 lean healthy men underwent a 38 h fasting period to induce ketosis. *D*-3HB kinetics and stereo isomers of muscle 3-hydroxybutyrylcarnitine were measured. Studies in mouse liver and muscle were performed to explore synthesis of *D*-3HB-carnitine, focusing on a hypothetical acyl-CoA synthetase (ACS) and succinyl-CoA oxoacid transferase (SCOT) pathway.

Results: Muscle *D*-3HB-carnitine was approximately 7.5 fold higher compared to *L*-3HB-carnitine. Muscle *D*-3HB-carnitine and *D*-3-HB turnover correlated significantly. The ACS pathway was active in liver and muscle homogenates though less than SCOT pathway that was only active in muscle homogenates.

Conclusions: We show that *D*-3HB-carnitine can be formed in muscle by mitochondrial SCOT and carnitine acyltransferase. Furthermore muscle *D*-3HB-carnitine correlates with the plasma turnover of *D*-3HB in lean healthy men during ketosis. Our data provide a newly identified alternative pathway of ketone body metabolism. The purpose of *D*-3HB-carnitine synthesis and its fate remain to be elucidated.

Introduction

The ketone bodies (KBs) *D*-3-hydroxybutyrate (*D*-3HB) and acetoacetate (AcAc) play an important role in the adaptation to fasting, serving as an important fuel for the central nervous system (1). KBs are produced mainly from fatty acids that are degraded through beta-oxidation within liver mitochondria. The resulting acetyl-CoA enters the tricarboxylic acid cycle (TCAC) or is channelled into the ketogenesis pathway (2;3). Via passive diffusion and active transport (monocarboxylate transporters 1, 2 and 4) *D*-3HB and AcAc will reach distant target tissues (e.g. central nervous system and skeletal muscle). *D*-3HB is oxidized to AcAc (2-4). AcAc then is coupled to CoA by succinyl-CoA 3-ketoacid(oxoacid) transferase (SCOT). The resulting acetoacetyl-CoA will yield two acetyl-CoAs that can be oxidized in the TCAC.

It has been suggested by at least two separate studies that *D*-3HB can be coupled to carnitine to form 3-hydroxybutyrylcarnitine (*D*-3HB-carnitine) (5;6). However, this raises a few questions. Although it has been described that *D*-3HB can be activated to *D*-3HB-CoA in rat liver and hepatoma cells, this has not been established in humans (7-9). Furthermore, it is unknown whether and how *D*-3HB-CoA can be coupled to carnitine resulting in *D*-3HB-carnitine.

This is in contrast to its generally known stereo isomer *L*-hydroxybutyryl-CoA (*L*-3HB-CoA), formed via the second step in the beta-oxidation of butyryl-CoA (10) i.e. hydration of crotonyl-CoA, thereby yielding the stereo-isomer *L*-3HB-CoA, which can be coupled to carnitine resulting in the formation of *L*-3HB-carnitine.

Although it was proposed in the earlier mentioned studies that the total amount of tissue hydroxybutyrylcarnitine was derived from *D*-3HB and carnitine, the quantative contributions of the *L* and *D* stereo isomers were not accounted for in these studies (5;6). This leaves the question which stereo isomers are present in vivo, unanswered.

In this study we investigated the relation between total *D*-3-HB turnover and muscle hydroxybutyryl-carnitine in 12 lean healthy men who underwent a 38 h fasting period to induce ketosis. *D*-3HB kinetics were measured using stable isotopes and the pancreatic clamp technique. Furthermore we assessed the quantitative contribution of the *D*- and *L*-stereo isomers to the total amount of muscle hydroxybutyrylcarnitine in the basal state, i.e. after 38 h of fasting and during a pancreatic clamp. The latter to study the changes in muscle *D* and *L*-3HB-carnitine upon a decrease in ketogenesis induced by mild hyperinsulinemia. We hypothesized total KB turnover rates to correlate with muscle *D*-3HB-carnitine. Additionally, we performed studies in mouse liver and muscle

homogenates to explore potential metabolic pathways responsible for the synthesis of *D*-3HB-carnitine; here we focused on SCOT and acyl-CoA synthetase (ACS). The activation of *D*-3HB and subsequent conversion to acylcarnitine would be a novel concept, offering an alternative pathway in ketone body metabolism.

Experimental procedures

Human pancreatic clamp studies

Data were included of twelve healthy lean male subjects who participated as controls in a study on obesity induced insulin resistance and KB metabolism (age: 24 (20 - 53) yrs; weight 73.8 (59.0 – 83.5); BMI 22.4 (18.9 – 24.7) kg/m^2) (Soeters, unpublished data). Criteria for inclusion were 1) absence of a family history of diabetes; 2) age 18–60 yr; 3) BMI 18-25 kg/m^2; 4) normal oral glucose tolerance test (OGTT) according to American Diabetes Association-criteria (11); 5) no excessive sport activities, i.e. < 3 times per week; and 6) no medication. Subjects were in self reported good health, confirmed by medical history, routine laboratory and physical examination. Written informed consent was obtained from all subjects after explanation of purposes, nature, and potential risks of the study. The study was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam.

Subjects started fasting at 2000 h two days before the study day to induce ketosis. On the study day, volunteers arrived at the metabolic unit of the Academic Medical Center at 0730 h. *D*[2,4-$^{13}C_2$]-3HB (>99% enriched; Cambridge Isotopes, Andover, USA) was used to study KB turnover. At T = 0 h (0800 h), blood samples were drawn for determination of background enrichments and a primed continuous infusion of *D*[2,4-$^{13}C_2$]-3HB (prime, 1.0 µmol/kg; continuous, 0.10 µmol/kg·min) was started and continued until the end of the study. After two hours of equilibration (38 h of fasting), 3 blood samples were drawn for KB enrichments and concentrations and 1 for glucoregulatory hormones and FFA. Then a pancreatic clamp was started at T = 2.5. Although the clamp included two steps (low dose insulin infusion at a rate of 4.5mU/m^2·min and 7.5mU/m^2·min respectively (Actrapid 100 IU/ml; Novo Nordisk Farma B.V., Alphen aan den Rijn, the Netherlands), only the last step is discussed in this report (referred to as "the clamp"). Glucose 5% was infused to maintain plasma glucose levels of 5 mmol/L. At the same time (T=2.5 h) infusion of somatostatin (250 µg/h; Somatostatine-ucb; UCB Pharma, Breda, the Netherlands) was started, as well as a glucagon infusion (1 ng/kg·min); Glucagen; Novo Nordisk, Alphen aan den Rijn, the Netherlands) to replace endogenous glucagon

concentrations throughout the clamp. Plasma glucose levels were monitored in 10 minute intervals. At the end of the clamp 5 blood samples were drawn for KB enrichments and concentrations, plasma glucose levels and 1 for glucoregulatory hormones and FFA.

Muscle biopsies were performed at the end of both basal state and clamp as described earlier (12).

Human plasma *D*-3HB kinetics and FFA

D-3HB samples were drawn in chilled sodium-fluoride tubes and directly deproteinized with ice cold perchloric acid 6% (1:1). *D*-3HB was measured spectrophotometrically using D-3-hydroxy-butyrate dehydrogenase (COBAS-FARA centrifugal analyzer, Roche Diagnostics, Almere, the Netherlands). *D*-3HB tracer tracee ratio's (TTR) were measured as follows: After centrifugation of the acidified KB samples, the supernatants were neutralized with KOH and $HClO_4$. Following centrifugation the supernatants were acidified with HCl and extracted twice with ethylacetate. The combined extracts were dried under nitrogen at room temperature. *D*-3HB was converted to its t-butyldimethylsilyl derivative with MTBSTFA and pyridine. The sample was injected into an Agilent 6890/5973 MSD gaschromotograph/mass spectrometer system (Agilent Technologies, Palo Alto, CA). Separation was achieved on a Varian (Middelburg, The Netherlands) CP-SIL 19CB column (30m x 0.25mm x 0.15µm). Selected ion monitoring (EI), data acquisition and quantitative calculations were performed using the Agilent Chemstation software. Ions were monitored at m/z 275 for unlabeled *D*-3HB and m/z 277 for *D*[2,4-$^{13}C_2$]-3HB. The enrichment in D-3HB was determined from standard curves of known enrichments injected in the same run.

D-3HB turnover (Ra) was calculated as the rate of isotope infusion (µmol/kg·min) divided by plateau tracer tracee ratios (TTR), hence *D*-3HB Ra was expressed as µmol/kg·min. Plasma glucose and FFA were measured as described previously (12). Plasma insulin levels were measured with an ultra sensitive human insulin RIA kit (Linco HI-11K, St. Charles, Missouri, USA; Detection limit 1.5 pmol/L).

Human muscle 3HB-carnitine and *D*- and *L*-3HB isomer measurements

Muscle hydroxybutyryl-carnitine levels were determined in freeze dried muscle specimens by tandem MS as described previously (13).

Muscle levels of the stereo isomers *D*- and *L*-3HB-carnitine were determined by high performance liquid chromatography (HPLC) tandem MS with minor modifications as described by Minkler et al (14).

Statistical analyses

All human data were analyzed with non parametric tests (Wilcoxon Signed Rank test). Correlations were expressed as Spearman's rank correlation coefficient (ρ). The SPSS statistical software program version 14.0 (SPSS Inc, Chicago, IL) was used for statistical analysis. Data are presented as median [minimum-maximum].

Experimental procedures muscle and liver homogenate studies

Mouse muscle (strain C57BL/6) was homogenized in PBS using an ultra turrax followed by sonication (3 times 40J). Liver was homogenized in PBS using sonication (2 times 40J) with constant cooling in an ice-water bath (4°C). Homogenates were centrifuged for 1 minute at 20xg. The incubation mixtures for ACS activity included, 100mM TrisHCl pH8, 1mM coenzyme A, 10mM $MgCl_2$, 10mM ATP, 5mM carnitine. The incubation mixtures for SCOT activity included: 100mM TrisHCl pH8.0, 0.25 mM succinyl-CoA, 5mM carnitine. For both mixtures (100 µL), we used 10mM *D,L*-3-hydroxybutyrate, 5mM *D*-3-hydroxybutyrate or 5mM *L*-3-hydroxybutyrate as substrate. The incubations were terminated by adding acetonitril containing the internal standards. After drying the samples with nitrogen, the reaction products were butylated using 1-butanol/acetylchloride (4:1). Samples were dissolved in acetonitril and analyzed by MS/MS. Carnitine acyltransferase activity was measured using the same tissue homogenates or purified pigeon breast carnitine acetyltransferase (CAT). The incubation mixtures (100 µL) included 100mM Tris.Cl pH8 and 5mM carnitine. As substrates, we used 220 µM *D,L*-3-hydroxybutyryl-CoA and 110 µM *D*-3-hydroxybutyryl-CoA. The *D*-3-hydroxybutyryl-CoA was prepared enzymatically from *D,L*-3-hydroxybutyryl-CoA, by using purified SCHAD that converts *L*-3-hydroxybutyryl-CoA into acetoacetyl-CoA. The *D*-3-hydroxybutyryl-CoA was purified using HPLC.

RESULTS

Human plasma *D*-3HB kinetics, FFA, glucose and insulin

Basal state values of *D*-3HB Ra, FFA, glucose and insulin are depicted in Table 1. Low dose insulin infusion significantly decreased plasma *D*-3HB levels as well as *D*-3HB Ra during the clamp compared to the basal state (Table 1). Likewise, plasma FFA decreased during the clamp compared to the basal state. Insulin infusion increased insulin levels as expected during the clamp compared to the basal state.

Table 1 Plasma measurements healhty lean volunteers

	basal state	clamp	
D-3HB (mmol/liter)	1.24 [0.30 - 2.03]	0.05 [0 - 0.40]	0.002
D-3HB Ra (µmol/kcal)	11.6 [6.9 - 22.4]	4.7 [1.3 - 9.0]	0.002
FFA (mmol/liter)	1.04 [0.72 - 1.21]	0.22 [0.06 - 0.48]	0.012
Glucose (mmol/liter)	4.1 [3.5 - 4.5]	5.0 [4.5 - 5.2]	0.002
Insulin (pmol/liter)	13 [6 - 27]	49 [26 - 69]	0.002

N = 12. Ra, rate of appearance; FFA, free fatty acids. Data are presented as median [minimum - maximum].

Human muscle 3HB-carnitine correlates with *D*-3HB Ra

Total (*D* and *L* isomers) muscle 3-HB-carnitine are depicted in Figure 1, panel A. Total muscle 3-HB-carnitine levers were lower after the clamp. We found a significant correlation in the basal state between total muscle 3-HB-carnitine and *D*-3HB Ra suggesting that the total amount of muscle 3-HB-carnitine is directly related to the 3-HB flux (Figure 1, panel B). Plasma FFA were found to correlate with total muscle 3-HB-carnitine (ρ 0.83, $P = 0.001$).

D-3HB-carnitine

Total muscle 3-HB-carnitine may consist of a *D* and *L* stereo isomer and since regular tandem MS does not discriminate between these two stereo isomers, we used HPLC-tandem MS to separate the two isomers. We found muscle *D*-3HB-carnitine to be approximately 7.5 fold higher compared to *L*-3HB-carnitine (Figure 1, panel C). Of interest, *L*-3-HB-carnitine could not be detected during the pancreatic clamp whereas *D*-3-HB-carnitine was significantly lower after the clamp. Results on muscle *D*-3HB-carnitine were missing in 2 subjects in the basal state and 3 during the clamp because measurements were below the detection limit. A significant positive correlation was found between muscle *D*-3HB-carnitine and the *D*-3-HB turnover in the basal state (Figure 1, panel D), however this was not the case during the clamp (clamp; ρ= 0.46, $P = 0.13$).

Synthesis of *D*-3-HB-carnitine in liver and muscle homogenates of mice

We hypothesized that the *D*-3-HB-carnitine could be formed by two alternative pathways. The first potential route is via acyl-CoA synthetase (ACS). *D*-3-HB can be activated to *D*-3HB-CoA by ACS activity, followed by the conversion to *D*-3HB-carnitine by a carnitine acyltransferase. This enzymatic reaction proceeds after the addition of ATP, free CoA and carnitine. The second hypothetical pathway is mediated by SCOT. SCOT transfers the CoA from succinyl-CoA to *D*-3HB, followed by the conversion to *D*-3HB-carnitine. Succinyl-CoA and carnitine are needed for this reaction. Both pathways were assessed

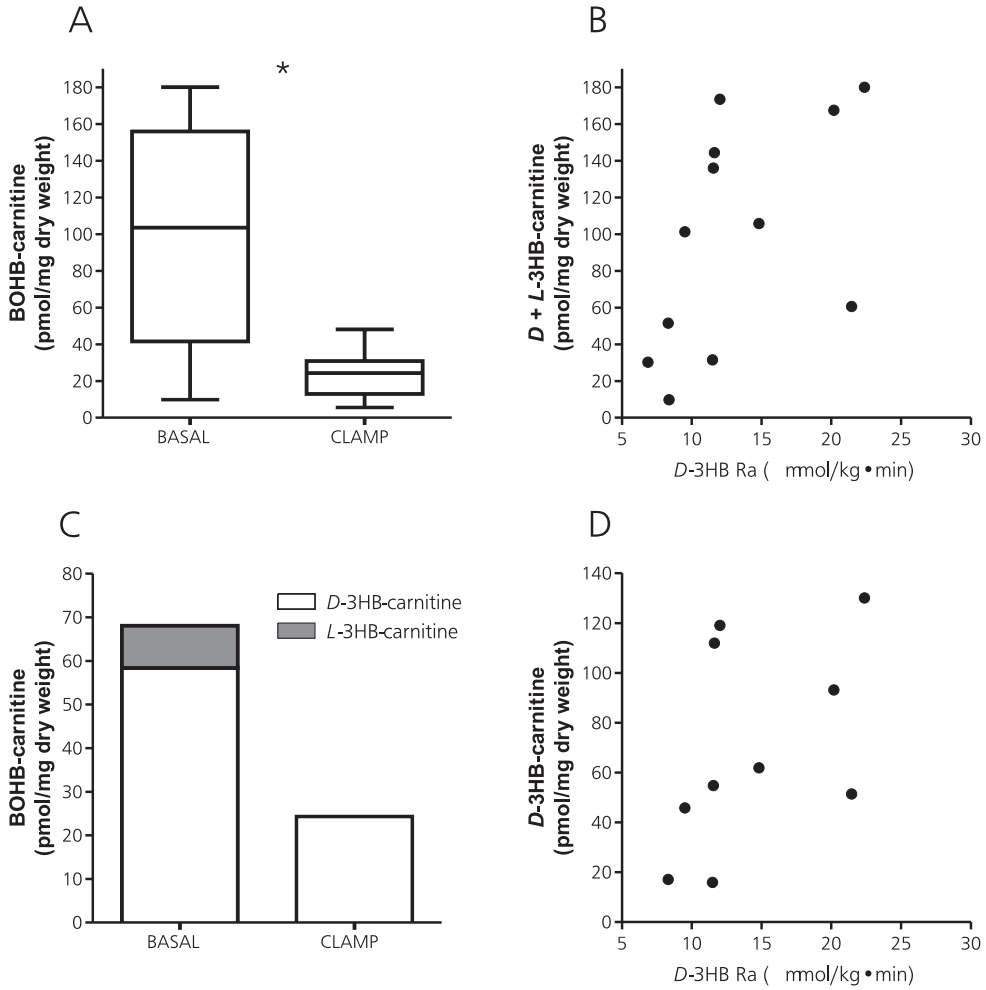

FIGURE 1 Panel A; Total amount of muscle 3-hydroxybutyrylcarnitine in the basal state and during the clamp * P = 0.002 (decrease in *D*-3-HB-carnitine during the clamp). Boxes and whiskers represent the median, minimum and maximum.
Panel B; Correlation of the total amount of muscle 3-hydroxybutyrylcarnitine with the *D*-3HB Ra in the basal state: $\rho = 0.74$, P = 0.006.
Panel C; Absolute contribution of *D*-3-HB-carnitine (empty bars) and *L*-3-HB-carnitine (grey bar) to the total amount of muscle 3-hydroxybutyrylcarnitine in the basal state (n = 10) and during the clamp (n = 9). Bars represent the median.
Panel D; Correlation of muscle *D*-3-HB-carnitine with the *D*-3HB Ra in the basal state: $\rho = 0.69$, P = 0.029 (n = 10).

in mouse muscle and liver homogenates by measuring the formation of 3-HB-carnitine from *D*- and *L*-3-HB. This method involves the measurement of two enzymatic steps, i.e. the activation of the 3-HB to 3-HB-CoA and the subsequent conversion to 3-HB-carnitine. The ACS pathway was active in liver and muscle homogenates (Figure 2, panels A and B). *L*-3-HB was converted into 3-HB-carnitine more efficiently than *D*-3-HB.

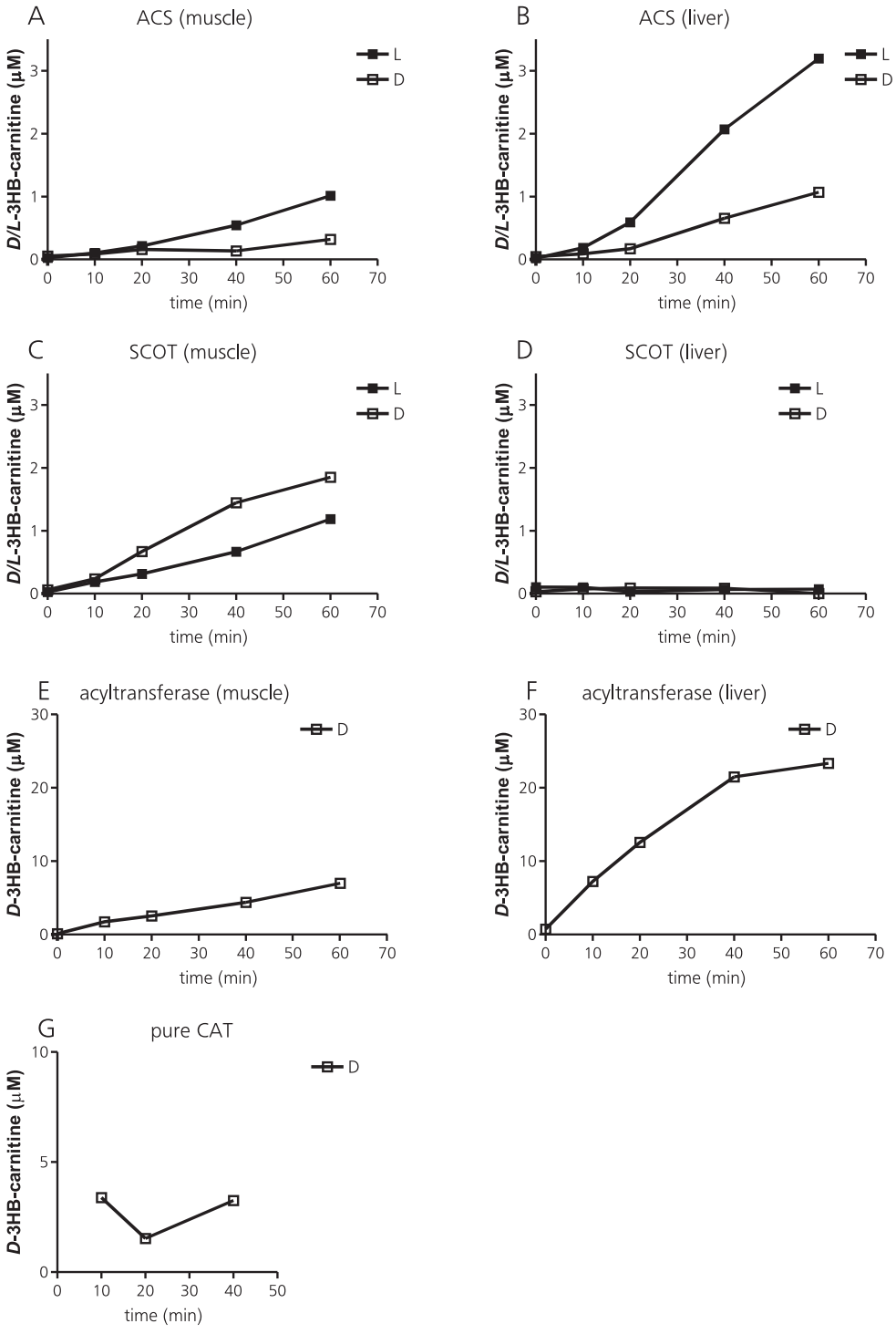

FIGURE 2 Panel A; Synthesis of *D/L*-3HB-carnitine in muscle homogenates of mice in presence of *D*-3HB, CoA, ATP and carnitine, catalyzed by acyl-CoA synthetase (ACS). *D/L*-3HB-carnitine are presented as open and closed boxes respectively.
Panel B; Synthesis of *D/L*-3HB-carnitine in liver homogenates of mice in presence of *D*-3HB, CoA, ATP and carnitine, catalyzed by acyl-CoA synthetase (ACS). *D/L*-3HB-carnitine are presented as open and closed boxes respectively.
Panel C; Synthesis of *D/L*-3HB-carnitine in muscle homogenates of mice in presence of *D*-3HB, succinyl-CoA and carnitine, catalyzed by succinyl-CoA 3-ketoacid(oxoacid) transferase (SCOT). *D/L*-3HB-carnitine are presented as open and closed boxes respectively.
Panel D; Synthesis of *D/L*-3HB-carnitine in liver homogenates of mice in presence of *D*-3HB, succinyl-CoA and carnitine, catalyzed by succinyl-CoA 3-ketoacid(oxoacid) transferase (SCOT). *D/L*-3HB-carnitine are presented as open and closed boxes respectively.
Panel E; Synthesis of *D*-3HB-carnitine in muscle homogenates of mice in presence of *D*-3HB-CoA and carnitine, catalyzed by acyltransferase.
Panel F; Synthesis of *D*-3HB-carnitine in liver homogenates of mice in presence of *D*-3HB-CoA and carnitine, catalyzed by acyltransferase.
Panel G; Synthesis of *D*-3HB-carnitine in Tris.Cl in presence of *D*-3HB-CoA and carnitine with purified pigeon carnitine acyltransferase (CAT).

Remarkably, the SCOT route was active in muscle homogenates only (Figure 2, panel C). With the addition of succinyl-CoA, *D*-3-HB was converted into 3-HB-carnitine more efficiently than *L*-3-HB. In liver this route was practically undetectable (Figure 2, panel D), which is consistent with the absence of SCOT activity in this tissue (3;15).

The second step in the formation of 3-HB-carnitine was hypothesized to be catalyzed by a carnitine acyltransferase. In liver and muscle homogenates this reaction proceeds as evident from the above described results. This was further evaluated in experiments were *D*-3HB-CoA was added to muscle and liver homogenates yielding *D*-3HB-carnitine. In liver homogenates this reaction was more efficient (Figure 2, panels E and F). Finally, we tested if this reaction is performed by CAT. Purified pigeon breast CAT was able to convert *D*-3HB-CoA to *D*-3-HB carnitine, although with low efficiency (Figure 2, panel G).

DISCUSSION

In this study we examined the relationship between the ketone body turnover and the presence of muscle *D*-3HB-carnitine and explored which isomer of 3-HB-carnitine is predominant in human skeletal muscle during ketosis induced by 38 h of fasting. We found *D*-3HB-carnitine to be the predominant 3HB-carnitine. Moreover, we show that muscle contains the full enzymatic machinery to synthesize *D*-3-HB-carnitine as shown in muscle homogenates in mice. This newly identified pathway involves an alternative metabolic route of ketone bodies in muscle.

We confirmed earlier reports on the association between muscle total 3-HB-carnitine and plasma KBs and FFA. It was shown by Hack et al that 3-HB-carnitine in adipose tissue microdialysis fluid in children correlated with plasma KBs after 24 h of fasting (6). Hack et al suggested that *D*-3HB could be coupled to carnitine in an unspecified enzymatic reaction as was observed for acetyl-CoA. An et al performed extensive acylcarnitine profiling in muscle samples of rodents and found that total muscle 3-HB-carnitine correlated with plasma FFA levels (5). They proposed muscle ketogenesis to be responsible for the amount of total muscle 3-HB-carnitine. However both studies did not validate their hypothesis by measuring both isomers of 3-HB-carnitine.

We used HPLC-MS/MS to separate *D*- and *L*-3-HB-carnitine and show for the first time that most of total muscle 3-HB-carnitine (approximately 88%) consists of *D*-3HB-carnitine after 38 h of fasting. Additionally, after 4 h of low dose insulin infusion, total muscle 3-HB-carnitine consisted entirely of *D*-3-HB-carnitine while the turnover rate of *D*-3HB had decreased by approximately 59%. Although we may not have detected low levels of muscle *L*-3-HB-carnitine, this suggests that beta-oxidation is decreased, thereby yielding less muscle *L*-3HB-carnitine. The metabolic pathway of *D*-3HB-carnitine is less clear.

Plasma *D*-3HB during short-term fasting is mainly derived from hepatic KB production (2;3). Therefore we measured the *D*-3HB production rate and found a correlation with muscle *D*-3HB-carnitine. This suggests that plasma *D*-3HB is transported to the myocyte where activation and subsequent coupling to carnitine occurs and fits with the assumption that the increased muscle *D*-3HB delivery during fasting exceeds muscle *D*-3HB oxidation analogous to what has been described previously for fatty acids in liver and muscle during fasting (16-18).

The existence of muscle *D*-3HB-carnitine and its relation to KB production in healthy lean volunteers prompts the question what metabolic pathway is responsible for the synthesis of *D*-3HB-carnitine. At least two reactions are critical: the activation of *D*-3HB to its CoA ester and secondly the exchange of CoA for carnitine. With this in mind we performed additional experiments in mice muscle and liver homogenates. We explored two possible metabolic pathways that may activate *D*-3HB: first we investigated whether *D*-3HB could be activated by ACS in the presence of ATP and CoA only. In muscle this was the case to some extend, however *L*-3HB appeared to be a better substrate yielding more *L*-3HB-carnitine (over 3-fold) compared to the *D*-isomer. Although *L*-3HB has been debated to be a natural occurring KB with low concentration (19-21), it was described in *in vitro* studies to be activated by a (cytosolic) ligase like ACS (7;22;23). The family of ACS includes short, medium, long and very long chain ACS (24). *L*-3HB could then be activated by either medium chain ACS or acetoacetyl-CoA synthetases (AACS), although this has

not been investigated in human muscle (25). The possibility of *D*-3HB to be activated was already suggested, although the catalyzing enzyme remained to be elucidated (8;9;23).

The second hypothetical pathway was activation of *D*-3-HB by SCOT. Indeed in muscle homogenates of mice we found significant activation of *D*-3-HB via this pathway. *L*-3-HB was less efficiently activated via this pathway. The robust activation of *D*-3HB in the presence of succinyl-CoA strongly suggests that SCOT (activating AcAc in the presence of succinyl-CoA) is involved in the synthesis of *D*-3-HB-carnitine in vivo in muscle (3;15). This was father supported by the finding that the addition of succinyl-CoA to liver homogenates did not result in a substantial amount of *D*-3HB-carnitine in accordance to the absence of SCOT in adult liver (3;15). In addition, AACS has been shown to decrease during fasting (26;27), at least in liver, making SCOT a better candidate to activate *D*-3HB in skeletal muscle.

The second step is the transfer of carnitine to *D*-3HB-CoA. In mouse muscle and liver homogenates, *D*-3HB-CoA was shown to be converted to *D*-3HB-carnitine. This reaction was more efficient in liver compared to muscle homogenates. Finally, we tested if this reaction is performed by CAT. Purified pigeon breast CAT was able to convert *D*-3HB-CoA to *D*-3HB-carnitine, although the with low efficiency suggesting that other carnitine acyltransferase might play a role (i.e. CPT2).

It is of interest where in the myocyte *D*-3HB is activated. Our results suggest that most of the activation occurs by SCOT within the mitochondrion (3;15). Also subsequent exchange of CoA for carnitine, possibly occurring via CAT or CPT2, is likely to be localized in the mitochondrium in humans (28).

A remaining question is whether the increase in muscle *D*-3HB-carnitine serves a purpose. First, *D*-3HB-carnitine can reflect true limitation of KB oxidation. It has been suggested for short chain acyl-CoAs (i.e. acetyl-CoA) that the coupling to carnitine provides an outlet to prevent mitochondrial accumulation (28;29). Finally, the increase in muscle *D*-3HB-carnitine may serve different other purposes. In the first place, it was shown that CoA levels in the rat heart, perfused with AcAc only, increase when carnitine is co-administered (30). This suggests that the coupling of *D*-3HB to carnitine may reduce CoA trapping. It was also suggested that the transfer of activated acylgroups to carnitine provides in transport needs between compartments and a reservoir of energy (28). Furthermore, ketosis can induce severe acidosis and it has been reported anecdotally that fasting in disorders with systemic carnitine deficiency can induce severe keto-acidosis suggesting that activation of *D*-3HB and subsequent transfer to carnitine may prevent keto-acidosis during fasting (31).

In conclusion we show in this study that *D*-3HB can be activated in muscle to its CoA ester presumably by mitochondrial SCOT. After activation, CAT catalyzes the transfer of CoA and carnitine thereby yielding *D*-3HB-carnitine. Hereby, *D*-3HB escapes immediate mitochondrial oxidation. Furthermore we have shown that muscle *D*-3HB-carnitine correlates with the plasma turnover of *D*-3HB in lean healthy men during ketosis induced by 38 h of fasting. Since this has not been explored in detail before, our data provide a newly identified alternative pathway of ketone body metabolism. The purpose of *D*-3HB-carnitine synthesis and its fate remain to be elucidated. Besides an inability to oxidize all formed *D*-3HB during ketosis, storage for intercompartmental transport, prevention of keto-acidosis or decreased CoA trapping may all occur.

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