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Short-Chain Fatty Acids Outpace Ketone Oxidation in the Failing Heart

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BACKGROUND: The failing heart is energy starved with impaired oxidation of long-chain fatty acids (LCFAs) at the level of reduced CPT1 (carnitine palmitoyltransferase 1) activity at the outer mitochondrial membrane. Recent work shows elevated ketone oxidation in failing hearts as an alternate carbon source for oxidative ATP generation. We hypothesized that another short-chain carbon source, short-chain fatty acids (SCFAs) that bypass carnitine palmitoyltransferase 1, could similarly support energy production in failing hearts.

METHODS: Cardiac hypertrophy and dysfunction were induced in rats by transverse-aortic constriction (TAC). Fourteen weeks after TAC or sham operation, isolated hearts were perfused with either the 4 carbon, ^{13}C-labeled ketone (D3-hydroxybutyrate) or the 4 carbon, ^{13}C-labeled SCFA butyrate in the presence of glucose and the LCFA palmitate. Oxidation of ketone and SCFA was compared by in vitro ^{13}C nuclear magnetic resonance spectroscopy, as was the capacity for short-chain carbon sources to compensate for impaired LCFA oxidation in the hypertrophic heart. Adaptive changes in enzyme expression and content for the distinct pathways of ketone and SCFA oxidation were examined in both failing rat and human hearts.

RESULTS: TAC produced pathological hypertrophy and increased the fractional contributions of ketone to acetyl coenzyme-A production in the tricarboxylic acid cycle (0.60±0.02 sham ketone versus 0.70±0.02 TAC ketone; $P<0.05$). However, butyrate oxidation in failing hearts was 15% greater (0.803±0.020 TAC SCFA) than ketone oxidation. SCFA was also more readily oxidized than ketone in sham hearts by 15% (0.693±0.020 sham SCFA). Despite greater SFCA oxidation, TAC did not change short-chain acyl coenzyme-A dehydrogenase content. However, failing hearts of humans and the rat model both contain significant increases in acyl coenzyme-A synthetase medium-chain 3 enzyme gene expression and protein content. The increased oxidation of SCFA and ketones occurred at the expense of LCFA oxidation, with LCFA contributing less to acetyl coenzyme-A production in failing hearts perfused with SCFA (0.190±0.012 TAC SCFA versus 0.3163±0.0360 TAC ketone).

CONCLUSIONS: SCFAs are more readily oxidized than ketones in failing hearts, despite both bypassing reduced CPT1 activity and represent an unexplored carbon source for energy production in failing hearts.

Key Words: cardiac ■ heart failure ■ ketones ■ metabolism ■ short-chain fatty acid

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Clinical Perspective

What Is New?

- The failing heart oxidizes short-chain fatty acids (SCFAs) more readily than ketones, with SCFA also displacing long-chain fatty oxidation to a greater extent.
- The SCFA butyrate has a higher affinity for entry into mitochondrial oxidation at the enzyme SCAD (short-chain acyl coenzyme A dehydrogenase) than does the ketone 3-hydroxybutyrate at the β-hydroxybutyrate dehydrogenase and then also through the respective downstream metabolic pathways for each substrate.
- Failing hearts of rats and humans have increased levels of ACSM3 (acyl coenzyme A synthetase medium-chain family member 3) enzyme, which can also oxidize SCFA to enhance butyrate oxidation.

What Are the Clinical Implications?

- Although ketones have been sought as a potential supplemental fuel to remedy the impaired oxidative metabolism of the failing heart, this study shows that failing hearts preferentially oxidize SCFAs over ketones, and SCFA may prove to be a more efficient energy source during pathological stress.
- Novel alterations in metabolic pathways favoring SCFA oxidation in the failing heart occur in patients with nonischemic cardiomyopathy.
- Circulating ketones are not a unique "superfuel" beyond the ability to bypass the inhibition of long-chain fat oxidation in the failing heart, as do SCFAs.

To meet its high energetic requirements, the beating heart is primarily reliant on the oxidation of long-chain fatty acids (LCFAs) for the generation of ATP.[1] With the development of heart failure, there is a reduction in the contribution of LCFA to oxidative ATP generation because of reduced activity of the major rate-limiting enzyme for LCFA oxidation, CPT1 (carnitine palmitoyltransferase 1), which results in reduced LCFA entry into the mitochondria.[1–6] Although compensatory glucose use occurs with hypertrophic remodeling, other maladaptive changes in glucose use pathways result in the inefficient oxidation of glucose and reduced energetic yield, further contributing to decompensation and energy starvation.[3,4] At present, great interest is emerging in ketones as an alternative substrate for oxidative energy metabolism.[7–9]

The decrease in CPT1 activity coincides with a shift in isoform expression in response to pathological stress on the heart. The normal adult myocardium coexpresses 2 CPT1 isoforms: (1) the muscle isoform, CPT1b, which is the predominant isoform in the adult cardiomyocyte and the lesser expressed liver isoform; and (2) CPT1a, which is more highly expressed in fetal cardiomyocytes. In response to pathological stress, within as early as 2 weeks and before any cardiac dysfunction, expression and content of the CPT1a isoform are elevated, whereas CPT1b message levels decline, with the CPT1b protein content either being reported to decline or not change.[2,10,11] Not only is there a reduction in CPT1 activity and flux through the enzyme–transporter complex, but there is also a reduction in LCFA activation to fatty acyl coenzyme-A (CoA) through ACSL1 (long-chain acyl CoA synthetase 1), which serves to exacerbate the limitation in fatty acyl supply for mitochondrial oxidative pathways.[2,12]

In contrast, ketones, and short-chain fatty acids (SCFAs) as well, are not dependent on CPT1 for mitochondrial entry nor ACSL1 for activation, but rather they cross the mitochondrial membrane through free diffusion, in the absence of any identified transport-mediated mechanism.[13,14] Thus, ketones bypass any inhibition of CPT1 in the heart, as would SCFA.[15] In the failing heart, there is an increase in the expression of the key enzymes regulating ketone oxidation and an increase in the circulating ketone concentration, leading to increased ketone use in both isolated failing hearts and in vivo human studies.[7,16,17] Such findings have led to the proposal that ketones are a key fuel source that could mitigate the reduced energy stores in the failing heart.[18] Recent studies clearly show elevated contributions of ketones to the mitochondrial oxidative pathway of the tricarboxylic acid (TCA) cycle in hypertrophied and failing hearts.[7,8] In animal models, this increase in ketone oxidation in pathologically stressed hearts has been shown to coincide with upregulation of the enzyme catalyzing the initial step of ketone oxidation, BDH1 (β-hydroxybutyrate dehydrogenase 1).[7,8,16] Whether increased ketone oxidation in the failing heart is an adaptive mechanism specific to elevated BDH1 expression and the oxidation of ketones or whether other fuels that bypass reduced CPT1 activity, such as SCFA, provide similar support and efficiency as alternate carbon-based substrates remains unknown.

In the present study, we directly compared the capacity of the ketone, 3-hydroxybutyrate (3-OHB) and the SCFA butyrate, both 4-carbon substrates, to fuel oxidative metabolism in the failing heart. As hypothesized, both short-chain carbon sources supplemented reduced LCFA oxidation in the failing heart. However, butyrate unexpectedly proved to be the preferred energy source in the heart versus 3-OHB, with a heightened preference in the hypertrophic heart. Although the results highlight the importance of identifying fuels that bypass the reduction in CPT1 activity and LCFA oxidation in the failing heart, the findings also show that BDH1 upregulation is not requisite to bypass CPT1 inhibition, elucidating the efficiency of SCFA oxidation

and an unexpected substrate preference by the failing heart. Thus, SCFAs were more efficient in bypassing CPT1 inhibition than ketone, and the findings reveal novel changes in cardiac metabolic pathways initiated by the failing heart to maintain carbon flux, evidence for which could also be found in the metabolic enzyme content of human hearts with nonischemic cardiomyopathy (NICM).

METHODS

Data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating procedures on reasonable request to the corresponding author.

Animal Model

Cardiac hypertrophy by chronic pressure overload for 14 weeks was induced by transverse aortic constriction (TAC) through a tantalum clip (Weck, Inc) placed around the transverse aorta of 32 male Sprague–Dawley rats (85–100 g) and set to an internal diameter of 0.56 mm, as described previously.[4,11] Male animals were studied on the basis of the need to match previously published protocols on which these studies are based. The sham groups (28 male Sprague–Dawley rats) underwent similar surgery without placement of the aortic band. Rats had free access to food and water while being housed under controlled temperature and lighting. Animals received either buprenex (0.1 mg/kg) or buprenex SR (1 mg/kg) before surgery and carprofen (5 mg/kg) once daily for 3 days postsurgery. Animals receiving buprenex also received twice daily buprenex (0.1 mg/kg) for 3 days postsurgery. Animals were intubated during surgery, and anesthesia was maintained through 0.5% to 2.0% isoflurane in 100% oxygen. All experimental procedures were approved by the institutional animal care and use committee at The Ohio State University.

Isolated Perfused Hearts

Fourteen weeks after TAC or sham surgery, animals were heparinized (1000 IU, intraperitoneal injection) and anesthetized (100 mg/kg pentobarbital, intraperitoneal injection). Hearts were excised and retrograde perfused with modified Krebs–Henseleit buffer (in mmol/L: 116.0 NaCl, 4.0 KCl, 1.5 $CaCl_2$, 1.2 $MgSO_4$, and 1.2 NaH_2PO_4) equilibrated with 95% O_2/5% CO_2 and containing 0.6 mmol/L of palmitate complexed to bovine serum albumin in a 3:1 molar ratio, 5 mmol/L of glucose, and either 1 mmol/L of sodium butyrate or sodium D-3-hydroxybutyrate (3-OHB). Buffer temperature was maintained at 37 °C. Hearts were situated in a 20-mm broadband nuclear magnetic resonance (NMR) probe within a 9.4-T, vertical bore (89-mm) NMR magnet, and a 2-minute ^{31}P NMR spectrum was acquired. The buffer was then switched for one with butyrate ([2,4-$^{13}C_2$] butyrate), 3-OHB ([2,4-$^{13}C_2$] 3-OHB), a mix of 0.5 mmol/L of butyrate and 0.5 mmol/L of 3-OHB in which only one of the substrates was ^{13}C labeled, or palmitate [2,4,6,8,10,12,14,16-$^{13}C_8$] with all ^{13}C at an enrichment of >99%. Hearts were perfused for 24 minutes until isotopic steady state was reached, at which time a second ^{31}P NMR spectrum was acquired, after which the hearts where rapidly frozen with liquid N_2-cooled tongs.

A water-filled latex balloon, connected to a force transducer, was fitted into the left ventricle and set to a diastolic pressure of 5 mm Hg. Left ventricular (LV)–developed pressure data were continuously acquired during perfusion with Powerlab (AD Instruments, Dunedin, New Zealand). Rate pressure product was calculated as heart rate × LV developed pressure, and mean peak rates of pressure development and relaxation were calculated from the first derivative of the LV developed pressure trace.

In Vitro NMR Spectroscopy

Perchloric acid extracts of frozen LV tissue from perfused hearts were lyophilized and reconstituted in 0.5 mL of deuterium oxide. High-resolution, proton-decoupled ^{13}C NMR spectra were acquired from in vitro samples with a 5-mm ^{13}C probe (Bruker Instruments, Billerica, MA). The relative contribution of butyrate, 3-OHB, and the LCFA palmitate to acetyl CoA entering the TCA cycle was determined as described previously from glutamate isotopomer and isotopologue analysis from detection of relative multiplet signals with the NMR signals from the glutamate 3- and 4-carbons.[19]

Acyl Carnitine Analysis

Acyl carnitines were isolated from frozen heart tissue, as described previously,[20] by homogenizing 25 mg of tissue in 0.1 mL of 100-mmol/L KH_2PO_4 and 0.25 mL of acetonitrile/isopropanol/methanol 3:1:1 (v/v/v). The homogenate was sonicated for 30 s and centrifuged for 10 minutes at 16 000*g* for 10 minutes. The supernatant was used for liquid chromatography tandem mass spectrometry analysis on the basis of established protocols with some modifications.[21] Briefly, supernatants were injected into a Vanquish UHPLC system connected to a TSQ Altis Triple Quadrupole mass spectrometry (electrospray ionization). Samples were separated on an Acclaim 120 C18 column at a flow rate of 0.4 mL/min heated to 30 °C. Mobile phases consisted of solvent A (10 mmol/L of ammonium formate, 0.1% formic acid, and 0.01% triethylamine in water) and solvent B (methanol). The following gradient was used: 0 minutes 0% B, 2 minutes 0% B, 12 minutes 50% B, 13 minutes 100% B, 15 minutes 100% B, 16 minutes 0% B, and 21 minutes 0% B. Labeled (^{13}C) and unlabeled (^{12}C) acyl carnitine species were identified by selective reaction monitoring. The precursor–product transitions that were used are listed in Table I in the Data Supplement for the identification of the individual acyl carnitine species.

Human Heart Tissue Collection

The protocol for surgical sampling of myocardium from patients was approved by the University of Utah Institutional Review Board. All patients provided written informed consent before inclusion.

Myocardial tissue was obtained from the LV apical core at continuous flow left ventricular assist device implant from 5 male patients. Transmural apical core biopsies also were acquired from 5 nonfailing donor hearts that were not allocated for human transplantation because of noncardiac reasons. Each transmural biopsy was immediately frozen and stored at –80 °C for metabolic enzyme expression

analysis. Patient characteristics are listed in Table II in the Data Supplement.

Metabolic Enzyme Expression

Protein expression was determined by Western blot and band intensities quantified by LI-COR Odyssey Fc and normalized to the expression of calsequestrin as a loading control, although mRNA levels were determined by quantitative reverse-transcription polymerase chain reaction in frozen heart tissue and normalized to S29.[11,12] Antibodies and primers used are listed in Table III and Table IV, respectively, in the Data Supplement).

Statistical Analysis

Data are presented as mean±SEM. Comparisons between 2 mean values were performed using the Student *t* test and among >2 mean values using 2-way ANOVA with a Tukey multiple comparison post hoc test. The statistical method used is indicated in each figure legend along with the individual n value and *P* values. Means were said to be statistically significant at $P<0.05$.

RESULTS

Sprague–Dawley rats (85–100 g at time of surgery) were subjected to chronic pressure overload through TAC surgery to induce decompensated pathological hypertrophy. Fourteen weeks after TAC or sham surgery there was a 35% increase in heart weight to tibia length (Figure 1A), and changes in the expression of the key enzymes regulating LCFA entry into the mitochondria were evident (Figure 1B), as reported previously.[10,11] TAC hearts displayed a 27% reduction in the expression of the predominant muscle isoforms of CPT1 and CPT1b and a 61% increase in the expression of the liver isoform, CPT1a. An increase in the expression of CPT1a in the heart is associated with a reduction in the rate of fatty acid oxidation and a net decrease in CPT1 activity and is consistent with previous reports of CPT1 expression in the failing heart.[4,10,11]

SCFA Butyrate Is a Preferred Substrate Over 3-OHB in Control and TAC Hearts

To access the relative contribution of SCFA and ketones to the TCA cycle, an indication of relative capacities of both to support mitochondrial oxidation, we investigated the oxidation of the 4-carbon SCFA butyrate and ketone 3-OHB in isolated hearts 14 weeks after TAC or sham surgery (control). Comparing a 4-carbon SCFA, butyrate, with the 4-carbon ketone, 3-OHB, allowed direct analysis of the oxidation of the 2 fuels, because both substrates yield 2 acetyl CoA molecules to fuel the TCA cycle (Figure 2A). Random distribution of animals resulted in a similar level of cardiac hypertrophy across each experimental protocol (Figure 2B). To provide comparison with previously published findings of ketone oxidation in hypertrophied hearts, isolated hearts were perfused for 24 minutes with medium that duplicated the previously published ketone concentration of 1 mmol/L of [2,4-^{13}C2] 3-OHB (^{13}C 3-OHB) or, for direct comparison, 1 mmol/L of [2,4-^{13}C2]butyrate (^{13}C butyrate), both in the presence of unlabeled glucose (5.0 mmol/L) and palmitate (0.6 mmol/L).[7,8] Analysis of glutamate isotopomers and isotopologues determined the contribution of each substrate to acetyl CoA formation and entry into the TCA cycle[19] (Figure 2C).

As established previously, cardiac hypertrophy coincided with an increase (17% increase versus 3-OHB sham, $P=0.0252$) in the relative contribution of 3-OHB to oxidative metabolism (Figure 2C and 2D).[7,8] The use of the SCFA butyrate was also increased in TAC hearts (16% versus butyrate sham, $P=0.0032$; Figure 2B and 2C), in contrast to the decline in LCFA oxidation observed in the failing heart.[1,5,11,22] It is striking that at both baseline and after the induction of hypertrophy, butyrate was oxidized to a greater extent than was 3-OHB. Butyrate was the preferred substrate relative to 3-OHB, contributing 15% more acetyl-CoA units entering the TCA cycle than did 3-OHB in both sham ($P=0.024$ butyrate sham versus 3-OHB sham) and TAC hearts ($P=0.0065$ butyrate TAC versus 3-OHB TAC; Figure 2D). When butyrate and 3-OHB were provided as a mix (0.5 mmol/L of butyrate with 0.5 mmol/L of 3-OHB) the cardiac preference for butyrate versus 3-OHB was greatly evident, with butyrate contributing 75% more acetyl-CoA units than 3-OHB TAC hearts (Figure 2E).

Increased Butyrate Oxidation in the Failing Heart Is Associated With an Increase in ACSM3 (Acyl CoA Synthetase Medium-Chain Family Member 3) Expression

Increased gene expression of the key dehydrogenase that regulates ketone oxidation, BDH1, has been reported in the myocardium of patients with heart failure, along with increased BDH1 expression and protein content in animal models of hypertrophy and heart failure.[7,16] In the present study, BDH1 mRNA and protein content were increased in hearts harvested at 14 weeks after TAC surgery relative to sham (Figure 3A and 3B). Conversely, the content of the key dehydrogenase regulating SCFA oxidation, SCAD (short-chain acyl CoA dehydrogenase), was not changed 14 weeks after TAC (Figure 3C and 3D). SCAD mRNA expression was found to be decreased with no change in protein expression. The protein expression of ACSM3 (acyl CoA synthetase medium-chain family member 3) was increased with TAC (Figure 3E). ACSM3 is a

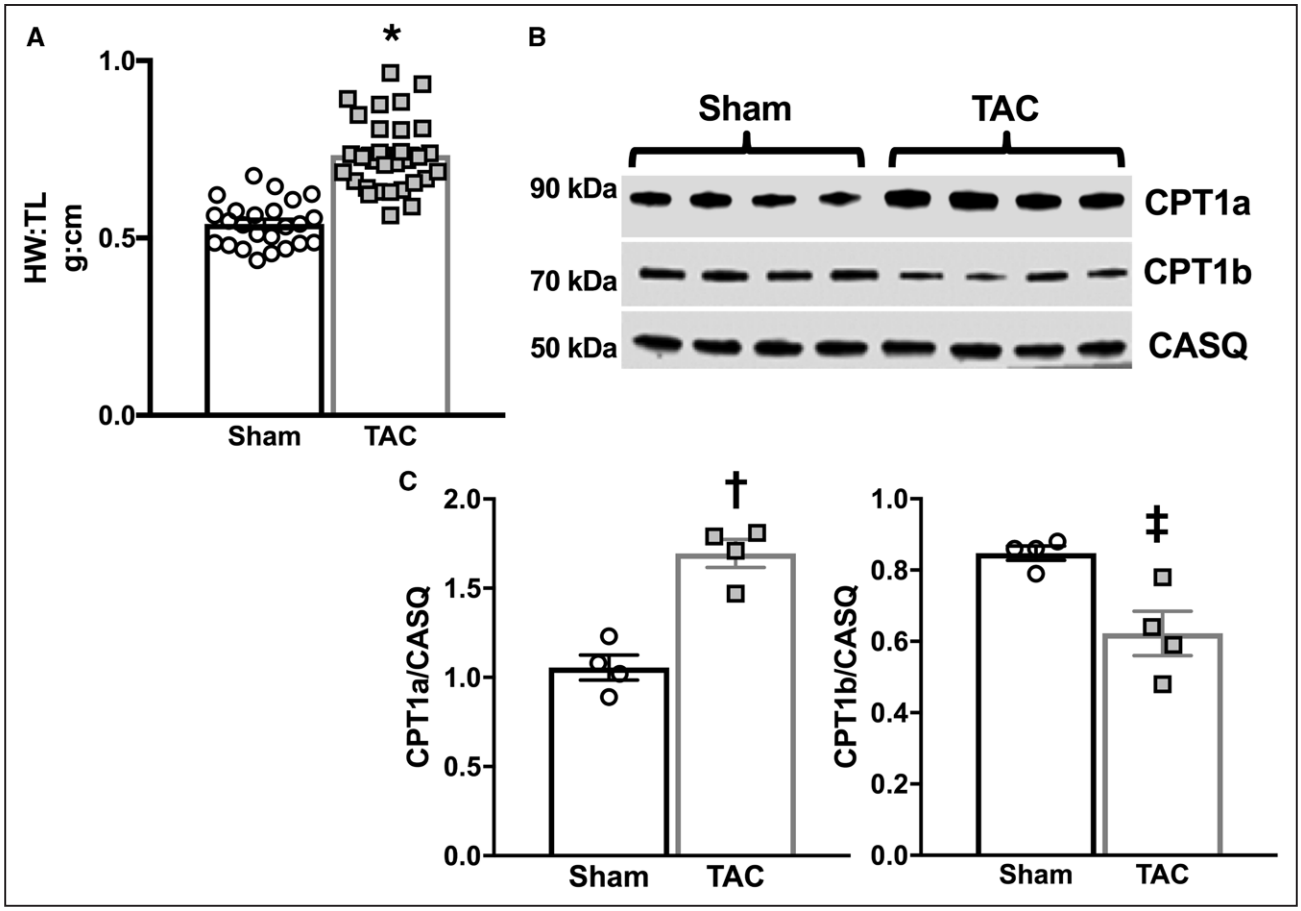

Figure 1. Hypertrophic response and CPT1 (carnitine palmitoyltransferase 1) expression in response to TAC.
A, HW:TL was measured in sham (n=24) and TAC hearts (n=31)14 weeks after sham or TAC surgery; **P*<0.0001 as determined by unpaired 2-tailed *t* test. **B,** CPT1a and CPT1b expression in unperfused sham or TAC hearts isolated 14 weeks after TAC or sham surgery. **C**, CPT1a and CPT1b protein expression normalized to CASQ (calsequestrin); †*P*=0.0009, ‡*P*<0.0137 versus sham as determined by unpaired 2-tailed *t* test. HW indicates heart weight; TAC, transverse aortic constriction; and TL, tibia length.

mitochondrial enzyme normally expressed at low levels in the heart that activates butyrate to butyryl CoA within the mitochondria.[23] Although a role for ACSM3 has been identified in cancer cell metabolism,[24] this is the first report of ACSM3 having a meaningful role in the failing heart.

Heart Failure in Humans Results in Increased ACSM3 Expression

To establish the relevance of our findings to the clinical patient population, we evaluated enzyme expression in human heart tissue from either donor hearts or patients with NICM, a pathology consistent with the TAC animal model. Somewhat surprisingly, we did not observe an increase in BDH1 mRNA expression (Figure 3F), nor was there a change in SCAD mRNA expression (Figure 3G). We also evaluated the myocardial protein content of both BDH1 and SCAD, as well as ACSM3 (Figure 3H–3K). In agreement with gene expression, the protein contents of BDH1 and SCAD were not different between donor hearts or patients with NICM. However, there was a significant increase in ACSM3 expression in NICM. ACSM3 in human heart failure or animal models of heart failure has not been investigated previously and reveals that the TAC-induced increase in ACSM3 is not species specific but also occurs in failing human hearts.

Butyrate Is Directed Toward β-Oxidation and Away From Pseudoketogensis in the Hypertrophic Heart

In addition to analyzing the contribution of short-chain carbon sources to the TCA cycle, we examined the myocardial content of short-chain acyl carnitine species in response to perfusion with either ^{13}C butyrate or ^{13}C 3-OHB. Carnitine esters for both fatty acyl and ketone intermediates are formed in the mitochondria and can serve as surrogates for the level of metabolic intermediates because they are more stable and are more readily quantifiable than short-chain fatty acyl CoA species in particular and are in equilibrium with oxidative intermediates (Figure I in the Data Supplement, Figure 4A). Liquid

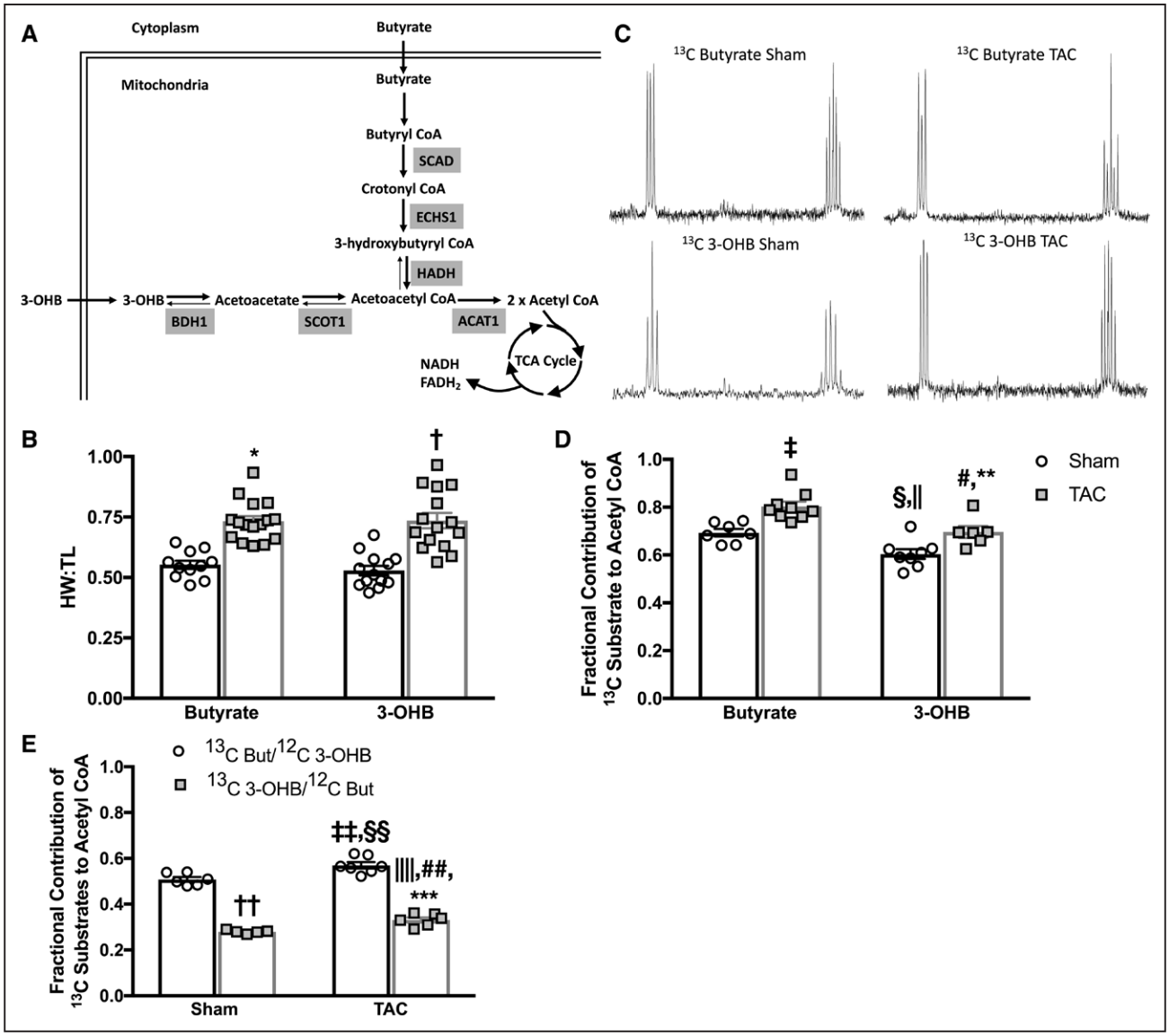

Figure 2. Fractional contribution of butyrate and 3-OHB to mitochondrial oxidation.
A, Model depicting the pathways for butyrate and 3-OHB oxidation in the heart. The role of enzymes ACAT1 (acetyl coenzyme A acetyltransferase 1), BDH1 (β-hydroxybutyrate dehydrogenase 1), ECHS1 (enoyl coenzyme A hydratase short-chain), HADH (3-hydroxyacyl coenzyme A dehydrogenase), SCAD (short-chain acyl coenzyme A dehydrogenase), and SCOT1 (succinyl coenzyme A:3-ketoacid coenzyme A transferase 1) in short-chain carbon metabolism is depicted. **B,** HW:TL as measured in isolated hearts at the end of perfusion with either butyrate (n=11 sham, n=16 TAC) or 3-OHB (n=13 sham, n=15 TAC) perfused hearts; *P<0.0001 versus butyrate sham, †P=0.0001 versus 3-OHB sham group via 2-way ANOVA and Tukey post hoc test. **C**, Representative spectra from end point enrichment analysis of glutamate ^{13}C enrichment in perfused hearts. **D**, Fractional contribution of either ^{13}C butyrate (n=7 sham, n=9 TAC) or ^{13}C 3-OHB (n=8 sham, n=6 TAC) to acetyl CoA; ‡P=0.0032 versus butyrate sham, §P=0.024 versus butyrate sham, ‖P<0.0001 versus butyrate TAC, #P=0.0065 versus butyrate TAC, **P=0.0252 versus 3-OHB sham via 2-way ANOVA and Tukey post hoc test. **E**, Fractional contribution of either ^{13}C butyrate (n=6 sham, n=5 TAC) or ^{13}C 3-OHB (n=7 sham, n=6 TAC) to acetyl CoA when provided as a 50:50 mix, ††P<0.0001 versus ^{13}C But/^{12}C 3-OHB sham, ‡‡P=0.0034 versus ^{13}C But/^{12}C 3-OHB sham, §§P<0.0001 versus ^{13}C 3-OHB/^{12}C But sham, ‖‖‖‖P<0.0001 versus ^{13}C But/^{12}C 3-OHB sham, ##P=0.0273 versus ^{13}C 3-OHB/^{12}C But sham, ***P<0.0001 versus ^{13}C But/^{12}C 3-OHB TAC, via 2-way ANOVA and Tukey post hoc test. But indicates butyrate; CoA, coenzyme-A; HW, heart weight; 3-OHB, 3-hydroxybutyrate; TAC, transverse aortic constriction; and TL, tibia length.

chromatography tandem mass spectrometry was used to determine the ^{13}C-enrichment of individual short-chain acyl carnitine species that were formed from either ^{13}C butyrate or ^{13}C 3-OHB in the isolated perfused hearts. As expected, ^{13}C butyrate led to enrichment of the butyryl carnitine pool (Figure I in the Data Supplement), although both ^{13}C butyrate and ^{13}C 3-OHB produced ^{13}C-enriched 3-hydroxybutyryl (C4OH) carnitine (Figure 4B). Butyrate led to formation of 2 species of ^{13}C C4OH, 3-hyxdroxybutyryl-L-carnitine (L-C4OH), which is in equilibrium with the β-oxidation intermediate 3-hydroxybutyryl CoA, and 3-hydroxybutyryl-D-carntine (D-C4OH; Figure 4A and 4B), which is in equilibrium with 3-OHB. D-C4OH is formed during butyrate perfusion by reversal of the reactions catalyzed by SCOT (succinyl CoA:3-ketoacid CoA transferase) and BDH1 leading to 3-OHB formation through

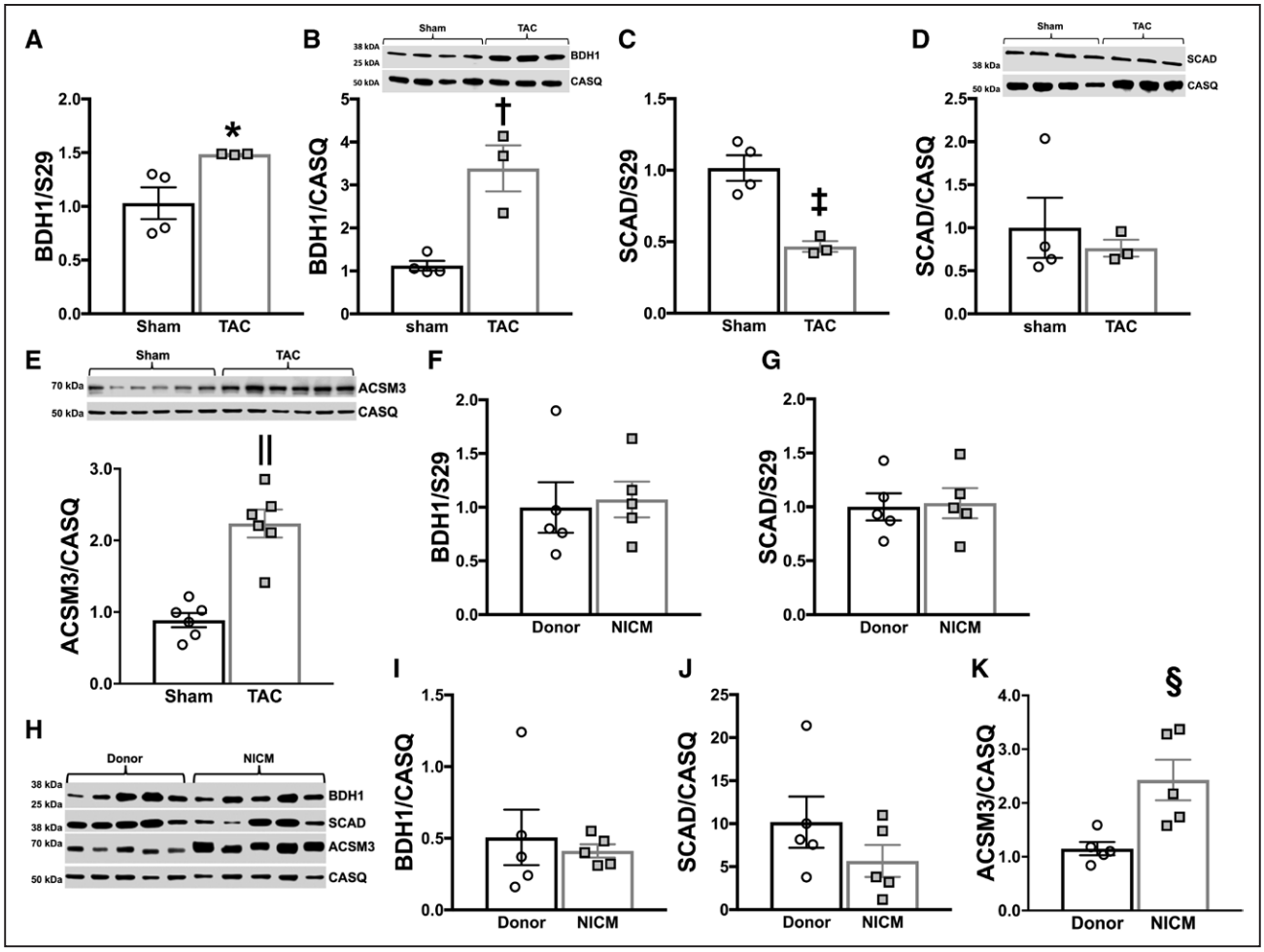

Figure 3. Expression of key enzymes regulating SCFA and ketone oxidation in the heart.
A, mRNA expression normalized to S29 of BDH1 (β-hydroxybutyrate dehydrogenase 1), **P*=0.0475 using a 2-tailed *t* test. **B**, BDH1 protein expression normalized to CASQ (calsequestrin), †*P*=0.0047 using a 2-tailed *t* test. **C**, mRNA expression normalized to S29 of SCAD (short-chain acyl coenzyme A dehydrogenase); ‡*P*=0.0041 using a 2-tailed *t* test. **D**, SCAD protein expression normalized to CASQ. **E**, ACSM3 (acyl coenzyme A synthetase medium-chain family member 3) protein expression normalized to CASQ, ‖*P*=0.0001 using a 2-tailed *t* test. mRNA expression normalized to S29 for BDH1 (**F**) and SCAD (**G**) from human donor hearts or hearts from patients with NICM. **H,** Protein expression for BDH1, SCAD, and ACSM3 measured in donor hearts or patients with NICM. Protein expression for BDH1 (**I**), SCAD (**J**), and ACSM3 (**K**) normalized to CASQ, §*P*=0.0125 using a 2-tailed *t* test. NICM indicates nonischemic cardiomyopathy; and SCFA, short-chain fatty acid.

a process that has been described as pseudoketogenisis.[25,26] Representative total ion chromatograms for ^{13}C C4OH reveal that, with the induction of cardiac hypertrophy, there is a significant reduction in the formation of ^{13}C D-C4OH in TAC hearts perfused with ^{13}C butyrate relative to sham hearts supplied with ^{13}C butyrate (Figure 4B and 4C), implying reduced pseudoketogenisis. The ratio of ^{13}C L-C4OH to the D isoform is significantly increased with the inclusion of ^{13}C butyrate to the perfusion medium (Figure 4C). Although the increase in this ratio is not surprising, because ^{13}C L-C4OH is in equilibrium with 3-hydroxybutyryl CoA, the further increase in TAC hearts supplied butyrate, relative to sham hearts supplied butyrate, suggests an increase in coupling between β-oxidation and TCA cycle activity and a reduction in reverse flux through both SCOT and BDH1.^{13}C 3-OHB led to very little formation of the oxidative intermediate (L-C4OH) at either baseline or in response to TAC, and a significant change in the ratio of 2 isoforms was not observed with TAC in the 3-OHB perfused hearts.

Contractile Performance and Energetic Status of the Failing Hearts Oxidizing Ketone or SCFA

To assess the potential of the alternative, 4-carbon fuel sources to acutely improve the energetic status of the failing heart, the ratio of phosphocreatine:ATP was examined as an index of the bioenergetic state of isolated hearts during perfusion with either butyrate or 3-OHB (Figure 5). Not surprisingly, a short perfusion protocol was not sufficient to impact the energetic status of the failing hearts (Figure 5A), and neither 3-OHB nor butyrate altered either the energetic status or the immediate mechanical function of the failing hearts for the duration of the perfusion protocols (Figure 5B–5D).

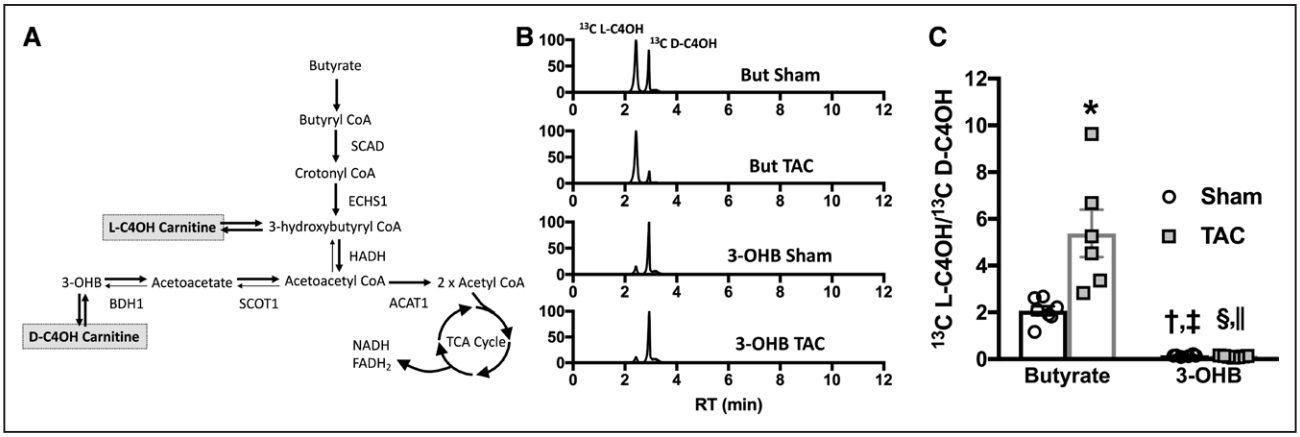

Figure 4. Changes in short chain acyl carnitine enrichment in hearts perfused with either butyrate of 3-OHB.
A, Sources of C4OH formed in equilibrium with oxidative pathway intermediates ACAT1 (acetyl coenzyme A acetyltransferase 1), BDH1 (β-hydroxybutyrate dehydrogenase 1), ECHS1 (enoyl coenzyme A hydratase short-chain), HADH (3-hydroxyacyl coenzyme A dehydrogenase), SCAD (short-chain acyl coenzyme A dehydrogenase), and SCOT1 (succinyl coenzyme A:3-ketoacid coenzyme A transferase 1). **B**, Representative total ion current chromatogram of ^{13}C enrichment of C4OH carnitine from either ^{13}C butyrate or ^{13}C 3-OHB. Two peaks are evident, L-C4OH and D-C4OH. **C**, The ratio of ^{13}C L-C4OH:^{13}C D-C4OH measured in hearts perfused with either ^{13}C butyrate (n=7 sham, n=6 TAC) or ^{13}C 3-OHB (n=6 sham, n=6 TAC); *P=0.0004 versus butyrate sham, †P=0.0438 versus butyrate sham, ‡P<0.0001 versus butyrate TAC, §P=0.0309 versus butyrate sham, ‖P<0.0001 versus butyrate TAC using a 2-way ANOVA and Tukey post hoc test. C4OH indicates 3-hydroxybutyryl carnitine; D-C4OH, 3-hydroxybutyryl-D-carnitine; L-C4OH, 3-hydroxybutryl-L-carnitine; 3-OHB, 3-hydroxybutyrate; and TAC, transverse aortic constriction.

Although there was not a change in the phosphocreatine:ATP ratio in response to either short-chain substrate, TAC hearts supplied with butyrate did not contain a significant difference in the ratio of pAMPK (phosphorylated AMP-activated protein kinase):AMPK (AMP-activated protein kinase) compared with sham hearts, although pAMPK:AMPK remained significantly increased in TAC hearts supplied with 3-OHB (Figure II in the Data Supplement). The data provide some evidence for an improvement in energetic status that is not evident in the ratio of phosphocreatine:ATP content; however other AMP-independent changes cannot be excluded.

Increased SCFA and Ketone Use Occurs at the Expense of LCFA

To verify that the use of short-chain carbon sources was supplementing reduced LCFA oxidation in the failing heart and to determine any influences of either ketones or SCFA on mitochondrial LCFA oxidation, the contribution of the LCFA, palmitate, to the TCA cycle was determined. Isolated hearts were perfused with 0.6 mmol/L of ^{13}C-labeled palmitate ([2,4,6,8,10,12,14,16-^{13}C8] palmitate) in the presence of either unlabeled butyrate or unlabeled 3-OHB. As reported previously, the LCFA contribution to mitochondrial oxidation in the TAC hearts was reduced compared with that of the sham heart (Figure 6A).[2,5,11,22] Furthermore, the reduction in LCFA oxidation in TAC hearts was offset by increased oxidation of both short-chain carbon sources (Figures 2D and 6B). However, as is consistent with the preferential oxidation of butyrate versus 3-OHB in both sham and TAC hearts, butyrate induced a greater reduction in LCFA oxidation than did 3-OHB in both sham and TAC hearts (Figure 6A). The data reveal that butyrate does indeed serve as an alternative fuel to counter reduced LCFA oxidation within the mitochondria in the failing heart and also displaces LCFA oxidation to a greater extent than does 3-OHB because of the SCFA being a more preferred oxidative fuel for ATP production than the ketone in both normal and failing hearts.

DISCUSSION

This is the first study to directly compare the ability of ketones and SCFA to fuel oxidative metabolism in the failing heart. The results reveal that SCFAs are a preferred energy source over ketones, and with the development of heart failure this relative preference for SCFAs is preserved. Although ketones represent a potential endogenous fuel source for the failing heart, SCFAs present a novel consideration through the capacity to more efficiently circumvent the inhibition of LCFA entry into mitochondria in the pathologically stressed heart. More significantly, the results reveal novel mechanistic insight into the remodeling that occurs during cardiac hypertrophy and highlight the degree to which the reduction in fatty acid entry through CPT1 is a primary limitation to the capacity of LCFA to fuel ATP production in the failing heart. Substrates that bypass CPT1 compensate for the deficit in LCFA transport into the mitochondria, allowing for previously unknown alterations in downstream pathways to facilitate entry of activated acyl units into the inner mitochondrial matrix and the TCA cycle of the failing heart.

The preference for SCFAs over ketones by the heart, when studied at equimolar concentrations of similar 4-carbon chain length SCFAs and ketone, has not been

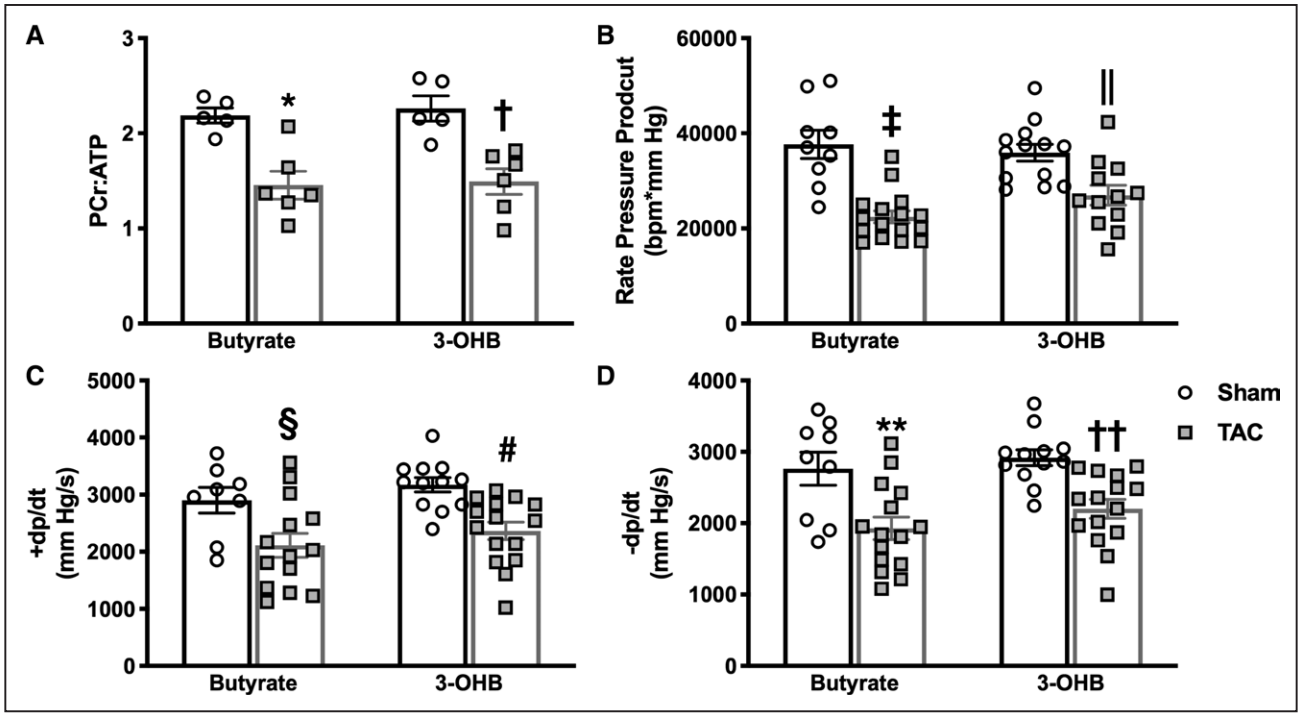

Figure 5. Energetic and contractile status of hearts perfused with butyrate or 3-OHB.
A, PCr:ATP ratio of hearts perfused with either butyrate or 3-OH 14 weeks after TAC or sham surgery, **P*=0.0044 and †*P*=0.007 versus sham group via 2-way ANOVA and Tukey post hoc test (n=5 for both sham groups, n=6 for both TAC groups). **B**, Rate pressure product. **C**, Maximum rate of pressure development (+dp/dt). **D**, Maximum rate of relaxation (–dp/dt) measured in left ventricle of isolated perfused hearts. ‡*P*<0.0001, §*P*=0.0089 versus sham; ‖*P*=0.0337, #*P*=0.0097 versus sham; ***P*=0.0042, ††*P*=0.0083 versus sham via 2-way ANOVA and Tukey post hoc test. For **B** through **D**, n=9 sham butyrate; n=13 sham 3-OHB; n=16 TAC butyrate; and n=12 TAC 3-OHB. 3-OHB indicates 3-hydroxybutyrate; PCr, phosphocreatine; and TAC, transverse aortic constriction.

demonstrated previously in either the normal or failing heart. The differences in use are not likely attributable to differences in extraction, because both substrates are understood to enter the cardiomyocyte via the monocarboxylate transporter.[14,27] Rather, these results suggest differences in the efficiencies of the 2 pathways for the generation of acetoacetyl CoA and acetyl CoA, once either of these substrates enters the mitochondria (Figure 2A). Unlike LCFA and the glycolytic end product, pyruvate, both ketones and SCFA are able to enter the mitochondria directly. Ketones first pass through BDH1, although SCFAs are activated to fatty acyl CoA through the ACSMs[28] and enter the first reaction of β-oxidation via SCAD.[7,14,27] We have elucidated for the first time a change in ACSM3 expression in failing hearts from both rat and human patient samples. Previously an impairment in LCFA activation to acyl CoA via ACSL1 was established by our group in both animal models and human heart failure.[12] The increase in ACSM3 is a previously unknown mechanism to address the reduction in fatty acid activation in the heart. However, accessing the full potential of this upregulation requires that fatty acids are able to enter the mitochondria independent of CPT1. Of note, ACSM3 shows broad substrate specificity (C4–C14) and in addition can activate LCFA. Past interest in medium-chain fatty acids and medium-chain triglycerides as a treatment strategy in cardiac disease has met with mixed success, potentially a function of study design. From the data obtained in this study, the ideal fatty acid makeup appears to be one in which specific fatty acids of chain lengths that are able to enter the mitochondria independent of CPT1 activity to fully access not only the increase in ACSM3, but also the reduction in pseudoketogenesis, which has not been documented previously in the failing heart.

An argument could be made that it is not surprising the SCFA would be a preferred energy source over ketones in normal hearts, because SCFA would share many of the enzymes involved in LCFA oxidation, acknowledged as the preferred energy source for the heart in vivo. The observation that this preference is maintained in the hypertrophic heart is surprising. Many of the enzymes involved in SCFA are targets of PPARα (peroxisome proliferator-activated receptor-α).[27,29–31] PPARα and many of its targets are downregulated in the failing heart, and the reduction in PPARα signaling is thought to be a primary event in reduction in fatty acid use in the failing heart.[11,32,33] The conclusion that must be drawn is that the increase in butyrate use is a compensatory response to the reduction in CPT1 activity induced by heart failure rather than a primary response to an increase in the oxidative enzyme expression that has been otherwise suggested for 3-OHB.[2,3,5,7,9,16,33,34] An increase in BDH1 expression was not observed in NICM. However, an increase in BDH1 gene expression has been reported in human failing myocardium,[16] but

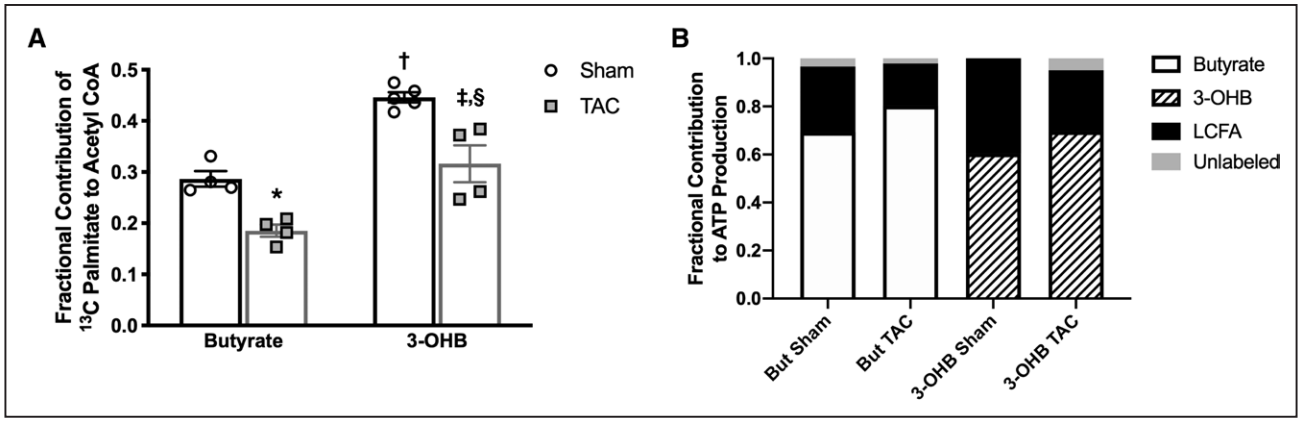

Figure 6. The contribution of the LCFA palmitate to mitochondrial oxidative metabolism in the hypertrophic heart perfused with either SCFAs or ketones.

A, The relative contribution of ^{13}C palmitate to acetyl CoA formation in the TCA cycle from in vitro NMR ^{13}C NMR; **P*=0.0181 versus butyrate sham, †*P*=0.0003 versus butyrate sham, ‡*P*=0.0029 versus butyrate TAC sham, §*P*=0.002 versus 3-OHB sham via 2-way ANOVA and Tukey post hoc test (n=4 sham butyrate, n=4 sham 3-OHB, n=5 TAC butyrate, n=4 TAC 3-OHB). **B**, A comparison of the contribution of either ^{13}C butyrate, ^{13}C 3-OHB, or ^{13}C palmitate to acetyl CoA formation in the TCA cycle by combining the data from **A** with that from Figure 2D. CoA indicates coenzyme-A; LCFA, long-chain fatty acid; NMR, nuclear magnetic resonance; 3-OHB, 3-hydroxybutyrate; SCFA, short-chain fatty acid; TAC, transverse aortic constriction; and TCA, tricarboxylic acid.

the increase was modest and BDH1 protein content has not been reported until now.

An increase in BDH1 is not necessarily requisite for ketone oxidation to be increased in the failing heart. Indeed, heart failure is known to coincide with increased circulating ketones,[16,17] and ketone extraction by the heart appears governed by the circulating concentration.[17] This is not the case for substrates that do not as readily cross the plasma membrane, such as glucose and LCFA. As the present findings suggest, the blockade that develops at the level of CPT1, on the outer mitochondrial membrane, would favor ketone oxidation even in the absence of additional metabolic alterations. Just as with SCFA, our data demonstrate that an increase in dehydrogenase expression may also not be requisite to produce augmented ketone, or SCFA, oxidation.

The therapeutic potential of ketones as an endogenous source of short-chain carbon to fuel the failing heart has already been discussed in previous studies.[9,16,34,35] We did not observe a functional improvement over the brief perfusion protocol in which short-chain carbon sources were provided, although AMPK phosphorylation was no longer significantly increased in TAC hearts perfused with butyrate. AMP-independent changes in AMPK phosphorylation can occur in response to factors not directly linked to energetic status. Previously, we and others have shown that the expression ACSL1 can alter AMPK phosphorylation, with AMPK phosphorylation increasing with ACLS1 overexpression and declining with the loss of ACSL1 expression.[12,36] It has been postulated that the dependence of AMPK phosphorylation on ACSL1 expression relates to the consumption of ATP by ACSL1,[36,37] however ACSM3 also consumes ATP to activate butyrate. As shown in this study, ACSM3 content is elevated in both the animal model of heart failure and in failing human hearts. The absence of any acute improvement in function is in line with another recent study in which ketones failed to induce any improvement in mechanical efficiency in failing hearts.[35] Contrasting our report is recent work by Horton et al,[8] showing infusion of ketones can ameliorate the functional decline in a model of pacing-induced heart failure in dogs. The latter study was not as acute as what occurred in our work or the work of Ho et al.[35] Thus, a chronic change in ketone availability or use appears to have an effect on the pathogenesis of heart failure, because both a ketogenic diet and overexpression of BDH1 attenuate the effects of TAC, although the loss of BDH1 expression leads to a more severe remodeling response.[8,9]

There has been interest in developing agents that increase circulating butyrate levels, particularly in the setting of cancer, because of the action of butyrate as a histone deacetylase inhibitor.[38] The butyrate prodrug tributyrin was capable of raising the circulating butyrate concentration to a mean value of 98 μmol/L in patients with advance solid tumors,[38] which is lower than the concentration used in the present study but within range of circulating ketone concentrations in patients with heart failure, under conditions of which ketones are argued to significantly contribute to cardiac energy production.[8,16,33] SCFAs are primarily produced by the gut microbiota at a ratio of 60:20:20 (acetate:propanoate:butyrate) and can reach a combined concentration of 100 mmol/L in the intestinal lumen; however, the circulating concentration of butyrate is significantly lower.[27,39] A diet high in fiber increases SCFA production by the gut and attenuates hypertrophic remodeling in response to hypertension; however, much of this protection has been attributed to an increase in acetate.[40] Conversely, the gut of patients with heart failure has been found to be depleted of

butyrate-producing bacteria,[41,42] and the loss of butyrate-producing bacteria in the gut of mice makes them more prone to cardiac remodeling in response to stress, which can be rescued by butyrate supplementation.[43,44] Butyrate in particular, but not acetate or propanoate, has been identified as a histone deacetylase inhibitor, leading to potent anti-inflammatory signaling, an effect that might actually be antagonized by ketones.[45] Therefore, SCFAs can display both class-specific and chain length–specific characteristics that remain to be fully understood. The present study focuses on comparing the acute effects of these short-chain carbon sources on mitochondrial metabolism. Future work may provide important insights by extending investigations to chronic studies to assess benefits of SCFAs generally, and butyrate specifically, on both energy provision and intracellular signaling through alternative pathways such as histone deacetylase inhibition.[38,46]

Apart from any potential therapeutic role as an alternative fuel that bypasses the potentially maladaptive reduction in LCFA oxidation in the failing heart, the primary finding of this study is the enhanced oxidation of butyrate within the mitochondria, over that of ketones, in the failing heart. Butyrate proved to be the more efficient substrate for oxidation within the TCA cycle compared with the ketone 3-OHB. The capacity of this SCFA to bypass the LCFA transport process of CPT1 revealed novel alterations in metabolic pathways favoring mitochondrial SCFA oxidation in the failing heart, changes validated in hearts of patients with NICM. Although we report no differences in the contractile performance of hearts provided either a ketone or an SCFA, in the presence of other physiological substrates, the implications of this preferential oxidation of butyrate are that ketones do not serve as a unique "superfuel" beyond availability in the circulation to bypass inhibition of LCFA at CPT1 in the failing heart and that the SCFA butyrate has a higher affinity for entry into mitochondrial oxidation at SCAD than does 3-OHB at BDH1 and then also through the respective downstream oxidative pathways for each substrate.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Materials

Data Supplement Figures I and II
Data Supplement Tables I–IV

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