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Human fetal intestinal epithelial cells metabolize and incorporate branched chain fatty acids in a structure specific manner[★]

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Abstract

Background—Branched chain fatty acids (BCFA) are constituents of gastrointestinal (GI) tract in healthy newborn human infants, reduce the incidence of necrotizing enterocolitis (NEC) in a neonatal rat model, and are incorporated into small intestine cellular lipids *in vivo*. We hypothesize that BCFA are taken up, metabolized and incorporated into human fetal cells *in vitro*.

Methods—Human H4 cells, a fetal non-transformed primary small intestine cell line, were incubated with albumin-bound non-esterified *anteiso*-17:0, *iso*-16:0, *iso*-18:0 and/or *iso*-20:0, and FA profiles in lipid fractions were analyzed.

Results—All BCFA were readily incorporated as major constituents of cellular lipids. *Anteiso*-17:0 was preferentially taken up, and was most effective among BCFA tested in displacing normal (*n*-) FA. The *iso* BCFA were preferred in reverse order of chain length, with *iso*-20:0 appearing at lowest level. BCFA incorporation in phospholipids (PL) followed the same order of preference, accumulating 42% of FA as BCFA with no overt morphological signs of cell death. Though cholesterol esters (CE) are at low cellular concentration among lipid classes examined, CE had the greatest affinity for BCFA, accumulating 65% of FA as BCFA. BCFA most

[★]Declaration. All authors declare no conflicts of interest.

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Contributors

L.L., V.W., A.W., K.S.D.K. J.T.B. conceived and designed the project. L.L., Z.W., H.G.P., C.X., P.L., X.S., V.W. conducted the experiments. L.L., H.G.P., P.L., K.S.D.K. J.T.B. analyzed and interpreted the data. All authors commented on the manuscript and approved the final version.

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effectively displaced lower saturated FA. *Iso*-16:0, *iso*-18:0 and *anteiso*-17:0 were both elongated and chain shortened by $\pm$ C2. *Iso*-20:0 was chain shortened to *iso*-18:0 and *iso*-16:0 but not elongated.

Conclusions—Nontransformed human fetal intestinal epithelial cells incorporate high levels of BCFA when they are available and metabolize them in a structure specific manner. These findings imply that specific pathways for handling BCFA are present in the lumen-facing cells of the human fetal GI tract that is exposed to vernix-derived BCFA in late gestation.

Keywords

lipids; phospholipids; diacylglycerol; triglycerides; free fatty acids; cholesterol esters; metabolism; intestine

Introduction

Branched chain fatty acids (BCFA) are a class of mostly saturated FA with one or more methyl branches, mostly located near the terminal methyl group (Supplementary Figure 1). They are synthesized in human sebaceous and meibomian glands and are components of sebum and meibum. Recently they have been recognized as bioactive dietary components commonly consumed by humans. Beef fat contains 1.6–1.9 wt% BCFA; dairy fat is about 2% BCFA and is dominated by *anteiso*-17:0 and *anteiso*-15:0 [1]. We have estimated that the mean BCFA intake in the U.S. is at least 500 mg/d; for comparison, intake of the well-studied DHA + EPA is about 100 mg/d [2].

Although BCFA are a daily dietary component, little is known about their digestion, metabolism or transport. In human studies, they are rarely reported among the FA in internal tissue. BCFA are a major component of the nutrition of late term human fetuses, ingested as components of vernix caseosa, the white waxy coating unique to humans, which contains about 30% BCFA of total FA. We estimate that only about 10% of the BCFA swallowed by the fetus appears in meconium, the waste material that accumulates in the rectum throughout gestation that is normally released only at the time of birth. Our data therefore indicate that 90% of ingested BCFA disappear in the developing neonatal GI tract [3]. In a neonatal rat pup model of necrotizing enterocolitis (NEC), dietary nonesterified BCFA reduced the incidence of disease to less than half the control level, while increasing the abundance of nascent bacteria species reliant on BCFA for membrane lipids. BCFA were found esterified into phospholipids of the ileum, serum, and liver in approximately equal proportions, indicating they are absorbed and transported, and not entirely consumed for energy [4]. Those animals were very immature neonates that are unlikely to be reliable models for human nutrient absorption and transport. The cellular fate of dietary BCFA in the lipids of any human cell type is virtually unknown [5].

H4 cells, a nontransformed (nonmalignant) epithelial cell line, were initially isolated from small intestine of fetuses at age of 20 to 22 weeks gestation and characterized by Sanderson *et al* [6]. H4 cells have been extensively used as cellular tools for studies of transportation of immunoglobulin in amniotic fluids [7], inflammation response [7, 8] and pathophysiology of NEC [9, 10]. We therefore chose these cells as a model for BCFA absorption and transport.

Here we test the hypothesis that BCFA be readily taken up and incorporated into cellular lipids in a structure specific way in fetal intestinal epithelial cells. H4 cells were incubated with *iso*-16:0, *anteiso*-17:0, *iso*-18:0, *iso*-20:0 individually or as a mixture, and BCFA incorporation into major lipid classes analyzed and compared. This restricted set of fatty acids were chosen because they are components of both vernix and of human and cow's milk.

Materials and Methods

Chemical

Iso-16:0, *anteiso*-17:0, *iso*-18:0 and *iso*-20, in FFA form were purchased from Larodan Fine Chemicals (Malmo, Sweden). Other chemicals, unless otherwise noted, were of HPLC/analytical grade and from Sigma-Aldrich (St. Louis, MO) or Burdick and Jackson (Muskegon, MI).

Cell culture

The non-transformed primary human fetal epithelial H4 cells are cultured according to a published protocol [10] with minor modifications. H4 cells were cultured in DMEM with 20% heat inactivated FBS, 1% glutamine, 1% Hepes, 1% sodium pyruvate, 1% non-essential amino acids, 0.2 U/ml human insulin and 50 U/mL penicillin, 50 µg/mL streptomycin at 37°C in a 5% CO₂ atmosphere. Cell culture media, FBS and reagents were obtained from Thermo Fisher Scientific (MA), Corning (NY), and Sigma-Aldrich (MO). Cells were grown in 25 cm² petri dishes before seeding. Analysis of FBS revealed trace BCFA levels near limits of detection (data not shown).

H4 cells incubation with BCFA

H4 cells were incubated with BSA bound BCFA (BCFA/BSA molar ratio at 3:1) prepared as described previously [10]. Briefly *iso*-16:0, *anteiso*-17:0, *iso*-18:0 and/or *iso*-20:0 were dissolved in ethanol by repeated heating at 60°C and vortexing to make a BCFA stock solution at 100 mM FA. BCFA stock (200 µl) was mixed with fatty acid free BSA in 1 × PBS (4.4% w/w) and incubated overnight at 37°C. BSA bound BCFA were filtered through 0.22 µm mesh and diluted to 0.1 mM by H4 media then added to cell media.

H4 cells ($n=2 \times 10^6$) were seeded at a density of $2 \times 10^5/\text{cm}^2$. After 24 h, cells were washed twice with 1 × PBS and then incubated with 0.1 mM *iso*-16:0, *anteiso*-17:0, *iso*-18:0 and *iso*-20:0 individually or a mixture of those four (0.4 mM in total). H4 cells incubated without BCFA were used as controls. After 24 h incubation, H4 cells were washed twice with 1 × PBS and harvested by trypsin. H4 cells used in present study were all at Passage 16.

Lipid extraction and analysis

Cell pellet lipids were extracted by the Bligh and Dyer method [11]. Neat lipid extracts were reconstituted with 100 µL chloroform and spotted onto a Silica gel G, 20 × 20 TLC plate (Analtech Inc., Newark, DE). A 2-step development protocol was used for separation of lipid fractions, in the same direction to insure adequate separation [12]. First, the plate was developed in chloroform: methanol: acetic acid: water (25:15:4:2, v:v:v:v) for a distance of

2.5 cm from the origin. After drying, a second development to 1 cm from the top of the plate was done with hexane: diethyl-ether: formic acid (80:20:2, v:v:v). Lipid bands were visualized within an iodine vapor tank. Visualized bands were carefully collected with known amount of heneicosanoic acid (21:0) as internal standard, then saponified and methyl-ester prepared with 14% BF₃ in methanol (Sigma-Aldrich, St. Louis, MO). Fatty acids methyl esters (FAME) were analyzed by GC-FID and identified by GC-MS/MS as described elsewhere [13]. Response factors using an external standard mixture was used applied to the raw data to correct for differential instrument response. The weight of total FA, as well as total BCFA, in each lipid fraction was calculated according to the corrected area of internal standard and calibrated.

Total FA and total BCFA in H4 cells were estimated by summing FA weights in each lipid fraction. BCFA uptake was adjusted for subtracting the small amount of BCFA in the control group from the BCFA treated groups. Uptake efficiency (%) was calculated by wt(BCFA uptake)/wt(BCFA in media) to establish whether cells take up BCFA in a structure specific manner.

Statistics

Data are expressed as mean $\pm$ SD. Pairwise t tests were performed to detect significant differences at the $p < 0.05$ level using Excel 2010 (Microsoft, Redmond, WA). SD reflect the means of two biological replicates except for the mix group.

Results

Morphological examination of H4 cells by light microscopy revealed no obvious differences between the untreated controls and the cells treated with BCFA, and no detectable difference in the number of floating cells were found. These observations indicate that H4 cells tolerate BCFA up to 0.4 mM. TLC revealed the presence of PL, MAG, DAG, FFA, TAG and CE bands as determined both by lipid standard and GC-MS/MS.

H4 cells readily take up BCFA

Uptake efficiency of individual BCFA by H4 cells was in the order of *anteiso*-17:0 (28%) > *iso*-16(17%) > *iso*-18:0 (10%) > *iso*-20:0 (7%) (Table 1), and all were significantly different from one another ($p < 0.05$).

The percentage of total BCFA of total FA is shown in Figure 1. As expected, BCFA incorporation into cell lipid follows the same trend as in Table 1, with *anteiso*-17:0 highest and the *iso* FA in a decreasing order. Remarkably, the percentage ranges from about 20% to 42% of FA with no apparent acute effect on the cell viability in the 24 h incubations.

BCFA distribution in lipids of H4 cells

We next investigated the lipid classes where BCFA appear. Figure 2 shows that BCFA enter all fractions, PL, TAG, FFA and CE, of mix-BCFA treated H4 cells at remarkably high levels (see also Supplementary Figure 2). CE has greater total BCFA in both treated and

control cells compared to the other fractions. Considering that PL and TAG are the predominant lipid classes in these cells, the greatest mass of BCFA appear in them.

Table 2 describes the relative distribution pattern of individual BCFA within H4 lipids. BCFA were predominantly incorporated into PL, and were at low levels in MAG and DAG. Patterns for *iso*-16:0, *anteiso*-17:0, and *iso*-18:0 were similar to one another with incorporation levels in order PL > TAG > CE > DAG > FFA > MAG. *Iso*-20:0 was lower in PL and much higher in FFA.

BCFA structural specificity in H4 cellular lipids

The PL fraction BCFA of H4 cell lipids for treated cells is presented in Figure 3. Control cells (not shown) had only *iso*-16:0 and *anteiso*-17:0 at trace level (both < 0.5%) in PL fraction. In treated cells, individual BCFA preference of incorporation into PL was in the order of *anteiso*-17:0 (32%) > *iso*-16:0 (28%) > *iso*-18:0 (20%) > *iso*-20:0 (6%), where *anteiso*-17:0 and *iso*-16:0 are not different but the means follow the same trend as in Table 1. A similar trend was found when the four competed as a mixture (Figure 3E). *Iso*-16:0 and *anteiso*-17:0 were chain elongated to *iso*-18:0 and *anteiso*-19:0 (see also Supplementary Figure 3), respectively, at levels of about 2% or less. Similarly, a small amount of *iso*-18:0 was chain shortened to *iso*-16:0. *Iso*-20:0 interconversion to other BCFA was most pronounced, with the chain shortened products *iso*-18:0 and *iso*-16:0 appearing at 63% and 20% of the abundance of *iso*-20:0.

TAG BCFA results are presented in Figure 4. TAG BCFA reached an even higher level than in PL, from 68% for *anteiso*-17:0 to 29% for *iso*-20:0 and in a similar order as for PL. CE BCFA are presented in Figure 5. Controls (not shown) accumulated around 7% BCFA. Again, *anteiso*-17:0 (71%, Figure 5B) was the most abundant BCFA and *iso*-20:0 (40%, Figure 5D) the least abundant. The 71% of total FA reached by *anteiso*-17:0 is the greatest level observed in this study for any BCFA in any fraction. Trends for FFA BCFA were opposite those of the other lipid classes, shown in Figure 6. *Iso*-20:0 was at 59%.

BCFA displace SFA

BCFA treatment predominantly caused a reduction of SFA% in H4 cells (Figure 7). The major SFA in cells, 16:0 and 18:0, both dropped proportion upon treatment with *iso*-16:0, *anteiso*-17:0, and *iso*-18:0 ($p < 0.05$) and a non-significant drop was observed for *iso*-20:0. *n*-20:0, a minor component, dropped in response to all BCFA. In contrast, BCFA did not significantly change total MUFA and PUFA in H4 cells.

Discussion

Our results show that BCFA are readily taken up, metabolized, and incorporated into cellular lipids in a structure specific manner in fetal enterocytes. *Anteiso*-17:0 emerged as the BCFA that was most effectively taken up and incorporated at the highest levels. Along with *anteiso*-15:0, *anteiso*-17:0 is the one of the two most abundant BCFA in dairy fat. The *anteiso* configuration confers substantial differences in biophysical properties compared to normal or *iso* FA. The melting points of C17 FA are 61, 60, and 37°C for *n*, *iso*, and *anteiso*,

respectively, while the phase transition temperatures of the di-C17:0-phosphatidylcholines are 49, 27, and 7°C for *n*, *iso*, and *anteiso* isomers, respectively [14].

No obvious morphological differences were found between the untreated controls and the cells treated with BCFA, nor was any difference noted in the number of floating cells. Previous data show that incubation of cells with (sub)millimolar SFA for 24h impairs cell signaling (e.g. via Akt) and induces ER stress and/or an apoptotic response [15–17]. In particular, incubation of cells with BCFA also causes apoptosis [18, 19]. In these studies, non-esterified BCFA were solubilized by non-ionic surfactants (Tween 80) and therefore were present as unbound fatty acids. As no normal (straight chain FA) controls were run, the effect may be due to high free fatty acid concentrations taken up by cells as intact lipid laden microparticles and may not be completely specific to the tested BCFA, isopentadecanoic acid (13-methyl tetradecanoic acid, 13-MTD). Indeed, unbound FA mediated cellular signaling events are reversed by the addition of albumin [20, 21]. BSA has seven binding sites with high to moderate affinity for fatty acids, 2–3 of which are high affinity with equilibrium constants on the order of 10^8 M^{-1} for palmitate [22]. The BCFA/BSA ratio in our experiments was 3:1, insuring that minimal unbound BCFA would be available to induce ER stress and/or an apoptotic response. We did not either test the cellular signaling events upon BCFA treatment or measure the ER stress and apoptotic response related makers, a topic for future studies.

BCFA have been long known to replace *cis* MUFA in microorganisms, presumably for modulation of membrane biophysical properties. A natural *E. coli* mutant lacking 9-desaturase activity failed to grow on saturated fats but had similar growth rates on oleic acid (18:1n-9) and *anteiso*-15:0 [23]. In the current study, BCFA replaced total SFA most effectively, the most abundant of which are 16:0 and 18:0. Replacement of the minor SFA 20:0 was similar. The pattern of replacement of *iso*-16:0 and *anteiso*-17:0 for the monounsaturates 18:1n-9 and 18:1n-7 was similar to normal SFA but not all significant. If BCFA and MUFA are serving similar biophysical roles, we might have expected their concentrations to be reciprocal rather than BCFA and normal SFA as we found. No significant replacement of BCFA for PUFA was found. The replacement of SFA rather than MUFA, at these high BCFA levels suggests that biophysical properties are less important, and that similar transporters and/or synthetic pathways handle both saturated normal and branched chain fatty acids. Presumably, BCFA and normal SFA compete for similar thioesterases, acyltransferases and/or cytidine diphosphocholine diacylglycerol choline phosphotransferases and MUFA have more orthogonal pathways. Further study is required to identify the underlying mechanisms.

The fetal GI tract processes a continuous input of swallowed amniotic fluid in late gestation, disposing of water and dissolved solutes via the kidneys. Vernix particles are the principle solid suspended in amniotic fluid, and vernix itself has been called the first solid meal of humans. BCFA constitute about 30% of the FA in vernix particles, a figure that is uniquely high among secreted human lipids [3]. Vernix is about 50% lipid by dry weight, and therefore the concentration of BCFA in the fetal lumen to which the enterocytes are exposed is likely in excess of 10% all solids. Our experiments with 0.1 mM (=100 μM) therefore were unlikely to be pharmacological compared to *in vivo* exposure. Although ingested

mostly as triglycerides in vernix, fetal dietary BCFA are extensively hydrolyzed in the stomach by lingual and gastric lipases to FFA and monoglycerides [24–26], prior to passing into the intestine. Enterocytes are therefore exposed *in vivo* to a multiphasic mixture with a high average concentration of FFA in micelles or a microemulsion. Our previous data indicate that BCFA at 500 μ M were non-toxic to Caco-2 cells, a transformed human epithelial colorectal adenocarcinoma cell line [27]. The high level of BCFA incorporation in our experiments, and apparently low toxicity *in vitro* suggests these cells are indeed highly tolerant and may well have evolved specific uses for BCFA at uniquely *in vivo* high exposures in the late term fetal gut. In contrast, *iso*-15:0 induces apoptosis in various human cancer cells with IC₅₀ ranging from 0.04 mM to 0.16 mM, including breast cancer (MCF-7), T-cell lymphomas (Jurkat and Hut78 cells), prostate cancer (E4 and DU145 cells), and hepatocarcinoma (NCI-H1688 cells)[18, 19]. In these studies, *iso*-15:0 was not solubilized by albumin, raising the possibility that the free fatty acid concentration was relatively high. Low toxicity in both transformed and non-transformed enterocytes and reported apoptosis in a variety of transformed cells lines suggests that enterocytes metabolize BCFA uniquely.

Molecular details of a role for BCFA in development have shown that BCFA-sphingolipids in the intestine are essential for the survival, foraging behavior and postembryonic development in *C. elegans*, and specifically for activation of TORC1 [5, 28, 29]. BCFA were independently essential for the vesicular trafficking pathway to maintain apical polarity of intestine [30], a critical link for activation of TORC1. Key proteins involved in BCFA biosynthesis from their precursor branched chain amino acids (BCAA), branched-chain ketoacid dehydrogenase complex, FA elongase ELO-5, and acyl CoA synthase ACS-1, are evolutionary conserved [5, 28], and the BCFA sphingolipid/TORC1 signaling pathway is conserved in mammals [29, 31–33]. Further research is required to understand how this pathway may operate in humans.

The *iso* BCFA taken up by H4 cells were in reverse order of chain length. Human fetuses ingest BCFA with chain lengths from C11 to C26 while meconium BCFA have chain lengths from C16 to C26 [3]. Our results are generally consistent with this trend, where the shorter chains preferentially are taken up leaving the longer chain BCFA in the lumen which would then find its way to meconium. A very old study reported that rats fed on *anteiso*-17:0 at 0.1 g/week had traces of *anteiso*-15:0 in adipose while it was not seen in controls [34], demonstrating *anteiso*-17:0 was chain shortened to *anteiso*-15:0. Our data provide direct evidence that fetal intestinal cells chain shorten BCFA by C2. We previously speculated that the preponderance of longer chain BCFA in meconium of human newborn could be ascribed to chain elongation of shorter chain BCFA, e.g. C13 to C15, *in vivo* [3]. Our data provide evidence that this process occurs in non-transformed fetal enterocytes.

In conclusion, our data show that H4 cells readily take up, metabolize, and incorporate BCFA into cellular lipids. Each of these processes is structure specific and specific to lipid fractions. The high BCFA concentration resulting from vernix entry into the fetal lumen is likely to have a role in supporting enterocyte growth and metabolism, and with it gut health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BCFA	branched-chain fatty acids
CE	cholesterol esters
DAG	diacylglycerol
FAME	fatty acid methyl esters
GI	gastrointestinal
MAG	monoacylglycerol
NEC	necrotizing enterocolitis
PL	phospholipids
SFA	saturated fatty acids
TAG	triacylglycerol

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Highlights

- Human fetuses among terrestrial animals are uniquely exposed to vernix caseosa lipids rich in saturated branched chain fatty acids (BCFA).
- Non-transformed human fetal enterocytes incubated with BCFA incorporate them into membrane phospholipids at levels from 35% to 64% in a structure specific manner, favoring *anteiso*-17:0.
- Though substantially lower melting than normal saturated FA, BCFA displaced 16:0 and 18:0 in preference to mono and polyunsaturates.
- These data predict that normal human fetal enterocytes incorporate high levels of BCFA *in vivo* when exposed to vernix in late gestation, which in turn may have physiological consequences.

Figure 1. Total BCFA levels (% w/w) in H4 cells

H4 cells were treated with an individual BCFA (*iso*-16:0, *anteiso*-17:0, *iso*-18:0, *iso*-20:0, n=2) or a mixture of four (n=1) or media only (control, n=2) for 24h. Means not sharing a common letter indicated a statistically significant difference between individual BCFA group ($p < 0.05$).

Figure 2. Total BCFA in each lipid fraction

BCFA% (w/w) in each lipid fraction was estimated in control (n=2) and mix group (n=1). BCFA most preferred in CE fraction as indicated both by control and mix group. Data not sharing a common letter indicated a statistically significant difference between different lipid class of control group ($p < 0.01$). Though there was no significant difference between CE and PL, and between CE and TAG, *iso*-16:0% in CE was higher than that in PL and TAG of control group ($p < 0.01$); *anteiso*-17:0% in CE was also higher than that in PL and TAG of control group ($p < 0.05$). No statistics was done between control group and mix group because there was only 1 replicate in mix group.

Figure 3. PL BCFA in H4 cells

Panel A, *iso*-16:0 group; Panel B, *anteiso*-17:0 group; Panel C, *iso*-18:0 group; Panel D, *iso*-20:0 group; Panel E, mix group. Only *iso*-16:0 and *anteiso*-17:0 could be detected at trace level (both <0.5%) in H4 cells without BCFA treatment (data not shown). BCFA's preference of incorporation into PL was in the order of *anteiso*-17:0 (32%) > *iso*-16 (28%) > *iso*-18 (20%) > *iso*-20 (6%) according to their levels in PL fraction. BCFA mixture treatment (E) confirmed it. n=2 except for the BCFA mix group (n=1). Data not sharing a common lowercase letter indicated a statistically significant difference between different BCFA% within panel ($p < 0.05$). Data not sharing a common capital letter indicated a statistically

significant difference between target BCFA% among panels ($p<0.05$). No statistics was done with mix group because there was only 1 replicate in mix group.

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Figure 4. TAG BCFA in H4 cells

Panel A, *iso*-16:0 group; Panel B, *anteiso*-17:0 group; Panel C, *iso*-18:0 group; Panel D, *iso*-20:0 group; Panel E, mix group. *Anteiso*-15:0, *iso*-16:0, *anteiso*-17:0 and *iso*-18:0 naturally occurred in TAG fractions of H4 cells with *anteiso*-17:0 level the highest (0.7%) (data not shown). TAG BCFA was greater than that in the PL fraction and their preference were in the order of *anteiso*-17:0 (68%) > *iso*-16 (46%) > *iso*-18 (44%) > *iso*-20 (29%) according to their levels in TAG fraction. Treatment with mixture led to a total BCFA level at 45% (E). n=2 except for the BCFA mix group (n=1). Data not sharing a common lowercase letter indicated a statistically significant difference between different BCFA%

within panel ($p < 0.05$). Data not sharing a common capital letter indicated a statistically significant difference between target BCFA% among panels ($p < 0.05$). No statistics was done with mix group because there was only 1 replicate in mix group.

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Figure 5. CE BCFA in H4 cells

Panel A, *iso*-16:0 group; Panel B, *anteiso*-17:0 group; Panel C, *iso*-18:0 group; Panel D, *iso*-20:0 group; Panel E, mix group. Naturally up to 7% total BCFA accumulated in CE (data not shown), which was the highest level compared to other lipid fractions. *Anteiso*-17:0 (71%, B) most abundant and *iso*-20:0 (40%, D) least abundant in CE. n=2 except for the BCFA mix group (n=1). Data not sharing a common lowercase letter indicated a statistically significant difference between different BCFA% within panel ($p<0.05$). Data not sharing a common capital letter indicated a statistically significant difference between target BCFA%

among panels ($p < 0.05$). No statistics was done with mix group because there was only 1 replicate in mix group.

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Figure 6. FFA BCFA in H4 cells

Panel A, *iso*-16:0 group; Panel B, *anteiso*-17:0 group; Panel C, *iso*-18:0 group; Panel D, *iso*-20:0 group; Panel E, mix group. *iso*-20:0, other than *anteiso*-17:0, most abundant in FFA fraction. The preference order of incorporation into CE was showing a reversal pattern compared with that in PL, TAG and CE fraction, *iso*-20 > *iso*-18:0 > *anteiso*-17:0 > *iso*-16:0. n=2 except for the BCFA mix group (n=1). Data not sharing a common lowercase letter indicated a statistically significant difference between different BCFA% within panel ($p < 0.05$). Data not sharing a common capital letter indicated a statistically significant

difference between target BCFA% among panels ($p<0.05$). No statistics was done with mix group because there was only 1 replicate in mix group.

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Figure 7. Displacement of normal FA by BCFA

n=2 except for the BCFA mix group (n=1). Means not sharing a common letter indicated a statistically significant difference between different BCFA treatments ($p < 0.05$). BCFA treatment primarily displaced saturated FA. *Iso*-16:0, *anteiso*-17:0 and *iso*-18:0 decreased total saturated FA ($p < 0.05$) and *iso*-20:0 followed the same trend (n.s.); individual normal FA followed the same trends. *Anteiso*-17:0 was most effective at displacing total normal saturated FA ($p < 0.05$) and displaced more normal MUFA and PUFA than other BCFA.

Table 1

Uptake efficiency of BCFA by H4 cells

BCFA	<i>Iso-16:0</i>	<i>Anteiso-17:0</i>	<i>Iso-18:0</i>	<i>Iso-20:0</i>
Uptake efficiency ^{<i>I</i>}	17.1 ± 1.6% ^{<i>a</i>}	27.5 ± 0.6% ^{<i>b</i>}	9.7 ± 0.3% ^{<i>c</i>}	6.8 ± 1.1% ^{<i>d</i>}

Total BCFA in H4 cells were estimated by summing FA weights in each lipid fraction. BCFA uptake was adjusted for subtracting the small amount of BCFA in the control group from the BCFA treated groups.

^{*I*}Uptake efficiency (%) = wt(BCFA uptake)/wt(BCFA added to media) × 100%.

^{*a,b,c,d*}Means not sharing a common letter are significantly different ($p < 0.05$). n=2

Table 2

BCFA incorporation into cellular lipids in H4 cells when incubated with specific BCFA.

BCFA (% or μg)	<i>Iso-16:0</i>	<i>Anteiso-17:0</i>	<i>Iso-18:0</i>	<i>Iso-20:0</i>
Total in cell (μg) ^I	21.6 $\pm$ 2.3 ^a	38.1 $\pm$ 0.8 ^b	13.4 $\pm$ 0.3 ^c	7.8 $\pm$ 1.4 ^d
PL (%)	63.5 $\pm$ 6.6 ^a	53.2 $\pm$ 3.0 ^a	62.8 $\pm$ 7.9 ^a	35.1 $\pm$ 35 ^b
MAG (%)	0.2 $\pm$ 0.0 ^a	0.3 $\pm$ 0.2 ^{ab}	0.9 $\pm$ 0.4 ^{ab}	0.5 $\pm$ 0.1 ^{bc}
DAG (%)	1.5 $\pm$ 0.1 ^a	1.8 $\pm$ 0.2 ^a	1.6 $\pm$ 0.3 ^a	1.8 $\pm$ 0.3 ^a
FFA (%)	0.9 $\pm$ 0.6 ^a	1.4 $\pm$ 0.3 ^{ab}	4.0 $\pm$ 1.3 ^b	24.5 $\pm$ 1.0 ^c
TAG (%)	25.0 $\pm$ 6.2 ^a	33.2 $\pm$ 4.6 ^a	21.3 $\pm$ 5.5 ^a	26.4 $\pm$ 4.4 ^a
CE (%)	8.8 $\pm$ 1.0 ^a	10.1 $\pm$ 1.0 ^{ab}	9.4 $\pm$ 0.3 ^a	11.7 $\pm$ 0.4 ^b

^ITotal BCFA in H4 cells were estimated by summing FA weights in each lipid fraction. PL *iso-16:0*% was calculated by wt(*iso-16:0* in PL)/wt(total *iso-16:0* in cell)×100% and so on.

^{a,b,c,d} Means not sharing a common letter indicated a statistically significant difference between individual BCFA ($p < 0.05$). n=2. Comparisons were made among individual BCFA within the same lipid classes.