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Branched short-chain fatty acids modulate glucose and lipid metabolism in primary adipocytes

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ABSTRACT

Short-chain fatty acids (SCFAs), e.g. acetic acid, propionic acid and butyric acid, generated through colonic fermentation of dietary fibers, have been shown to reach the systemic circulation at micromolar concentrations. Moreover, SCFAs have been conferred anti-obesity properties in both animal models and human subjects. Branched SCFAs (BSCFAs), e.g., isobutyric and isovaleric acid, are generated by fermentation of branched amino acids, generated from undigested protein reaching colon. However, BSCFAs have been sparsely investigated when referring to effects on energy metabolism. Here we primarily investigate the effects of isobutyric acid and isovaleric acid on glucose and lipid metabolism in primary rat and human adipocytes. BSCFAs inhibited both cAMP-mediated lipolysis and insulin-stimulated *de novo* lipogenesis at 10 mM, whereas isobutyric acid potentiated insulin-stimulated glucose uptake by all concentrations (1, 3 and 10 mM) in rat adipocytes. For human adipocytes, only SCFAs inhibited lipolysis at 10 mM. In both *in vitro* models, BSCFAs and SCFAs reduced phosphorylation of hormone sensitive lipase, a rate limiting enzyme in lipolysis. In addition, BSCFAs and SCFAs, in contrast to insulin, inhibited lipolysis in the presence of wortmannin, a phosphatidylinositide 3-kinase inhibitor and OPC3911, a phosphodiesterase 3 inhibitor in rat adipocytes. Furthermore, BSCFAs and SCFAs reduced insulin-mediated phosphorylation of protein kinase B. To conclude, BSCFAs have effects on adipocyte lipid and glucose metabolism that can contribute to improved insulin sensitivity in individuals with disturbed metabolism.

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Introduction

A diet rich in dietary fibers influences the regulation of weight and appetite control as well as energy homeostasis.[1-3] These effects are most likely mediated by the short-chain fatty acids (SCFAs), generated through colonic fermentation of dietary fibers.[4-11] The most abundant SCFAs, namely acetic acid, propionic acid and butyric acid, appear to have anti-obesity properties in both animal models and human subjects when orally distributed.[12-21] For example, a high fat diet supplemented with acetic acid, propionic acid or butyric acid improved both insulin sensitivity and protected against weight gain in animal models.[18,19] Furthermore, inulin-propionate, a dietary fiber combined with the ester of propionic acid, reduced energy intake, accumulation of intra-abdominal adiposity and lipid content in the liver in obese human individuals.[20]

Branched SCFAs (BSCFAs), e.g. isobutyric and isovaleric acid, are generated by fermentation of branched amino acids, valine, leucine and isoleucine,[22] generated from undigestible protein reaching colon.[23,24] However, an increase of isobutyric acid has also been demonstrated after ingestion of certain dietary fibers, such as polydextrose.[25] To the best of our knowledge, less is known regarding the role of gut-derived BSCFAs in the regulation of metabolism. Nevertheless, a recent study showed that a diet composed of brown beans increased colonic production of isobutyric acid and propionic acid, which was associated with lowered glucose and insulin concentrations in the blood as well as increased production of the satiety hormone pancreatic peptide YY (PYY).[26] Furthermore, by supplementing the drinking water with leucine, a branched amino acid, to mice fed a high-fat diet has been shown to improve glucose tolerance and insulin signaling as well as decrease inflammation in adipose tissue.[27] In obesity, adipose tissue shows a number of structural, morphological and functional alterations associated with a deteriorated fat storage capacity, an imbalance in the circulating levels of fatty acids and adipose tissue-derived hormones as well as pro-inflammatory cytokines that promote insulin resistance.[28-31] Thus,

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adipose tissue is a key target tissue for prevention of type 2 diabetes. We and others have previously demonstrated health beneficial effects of SCFAs on adipocyte function, involving effects on lipolysis, lipogenesis and glucose uptake.[32-38] BSCFAs have not been associated with outcomes on host health as is the case for SCFAs and specifically, effects of BSCFAs have not been reported when referring to effects on adipocyte metabolism. In the current study, we investigate whether the 2 BSCFAs isobutyric acid and isovaleric acid have the ability to modulate adipocyte function.

Results

Branched short-chain fatty acids inhibit isoproterenol-stimulated lipolysis in a PI3-kinase and PDE3 independent manner

Adipocyte lipolysis is mediated by hormones that increase cAMP, leading to protein kinase A (PKA)-mediated phosphorylation and activation of hormone sensitive lipase (HSL), a rate limiting enzyme in hormone-stimulated lipolysis.[30] The effects of the BSCFAs isobutyric acid and isovaleric acid, and the SCFA acetic acid on basal and isoproterenol-stimulated lipolysis were investigated in primary rat adipocytes. As shown in Table 1, BSCFAs and acetic acid inhibited isoproterenol-stimulated lipolysis, which was associated with reduced phosphorylation of HSL (Fig. 1, Table 2).

Table 1. BSCFAs and acetic acid inhibit lipolysis in rat adipocytes. Lipolysis was measured after 30 minutes of stimulation without (BASAL) or with 30 nM isoproterenol (ISO) in the absence (CTRL) or presence of isobutyric acid (I-BA), isovaleric acid (I-VA) and acetic acid (AA). The values for I-BA, I-VA or AA are related to respective CTRL (set to 1) in BASAL and ISO condition. The mean ± SD (n = 3-5) were used and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. [a]8.4-fold, [b]8.3-fold, [c]6.4-fold increase in ISO-stimulated lipolysis compared to BASAL.

		1 I-BA		3 I-BA		10 I-BA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	0.74	0.57	0.90	0.52	0.60*	0.31
ISO	1[a]	1.07	0.07	0.98	0.12	0.39**	0.26
		1 I-VA		3 I-VA		10 I-VA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	1.73*	0.18	0.95	0.02	0.75	0.27
ISO	1[b]	1.10*	0.03	1.05	0.03	0.50**	0.05
		1 AA		3 AA		10 AA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	1.54*	0.29	0.81	0.21	1.16	0.42
ISO	1[c]	1.05	0.03	0.93	0.09	0.33***	0.08

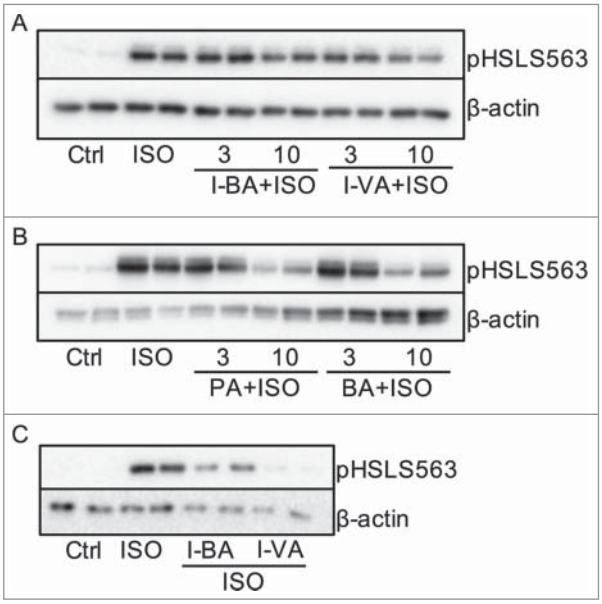

Figure 1. BSCFAs and SCFAs inhibit ISO-potentiated phosphorylation of HSL in primary rat and human adipocytes. In A-B, rat adipocytes were stimulated without (Ctrl) and with isoproterenol (ISO) in the presence or absence of 3 and 10 mM isobutyric acid (I- BA), isovaleric acid (I-VA), propionic acid (PA) and butyric acid (BA) for 10 minutes (n = 3). In C, human adipocytes were stimulated with ISO in the presence or absence of 10 mM I-BA or I-VA for 10 minutes (n = 3). Homogenates were subjected to immunoblot analysis and membranes were probed with antibodies against pHSLS563 and β-actin. Representative blots are shown.

Insulin mediates its antilipolytic effect to a large extent via a phosphatidylinositide 3-kinase (PI3 kinase)- and protein kinase B (PKB)-dependent activation of the cAMP-degrading enzyme phosphodiesterase (PDE) 3B.[39,40] However, as shown in Figure 2, the PI3 kinase- selective inhibitor wortmannin and the PDE3-selective inhibitor OPC3911 did not prevent the antilipolytic effect of either branched or non-branched SCFAs on isoproterenol-mediated lipolysis, whereas the antilipolytic effect of insulin was blocked by the inhibitors. To get the human perspective, we also studied lipolysis in adipocytes isolated from subcutaneous adipose tissue of human donors. BSCFAs and SCFAs reduce phosphorylation of HSL whereas significant inhibition of lipolysis was obtained with SCFAs (Fig. 1, Fig. 3, Table 2).

Branched short-chain fatty acids inhibit basal and stimulated lipogenesis

The effect of BSCFAs and acetic acid on lipogenesis was studied in primary rat adipocytes. Both basal and insulin-stimulated lipogenesis, measured as the incorporation of [^{3}H]-labeled glucose into cellular lipids, were inhibited by isobutyric and isovaleric acid, although to a lesser extent for isovaleric acid in the basal condition (Table 3). Also, acetic acid resulted in inhibition of lipogenesis (Table 3).

Isobutyric acid potentiates insulin-stimulated glucose uptake

The effect BSCFAs as well as acetic acid on glucose uptake was studied in primary rat adipocytes. As shown in Table 4, isobutyric acid induced a small, but significant potentiation of both basal and insulin-stimulated glucose uptake. The highest concentration of isovaleric acid inhibited basal glucose uptake, while no effect on insulin-stimulated glucose uptake was observed, which was also the case for acetic acid.

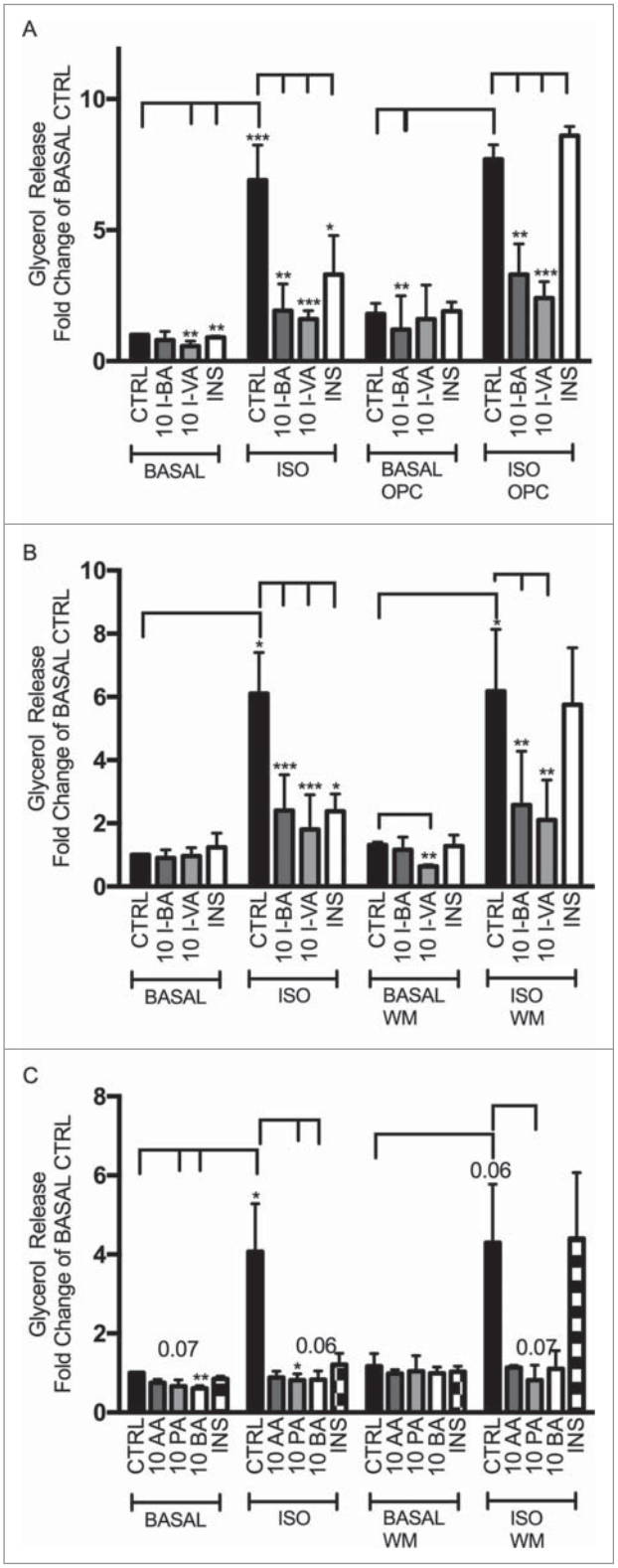

Table 2. BSFAs and SCFAs reduce phosphorylation of HSLS563 in primary human and rat adipocytes. Rat adipocytes were stimulated without (CTRL) or with ISO in the absence or presence of 3 and 10 mM isobutyric acid (I-BA), isovaleric acid (I-VA), propionic acid (PA) and butyric acid (BA) for 10 minutes. Human adipocytes were stimulated without (CTRL) or with ISO in the absence or presence of 10 mM I-BA or I-VA for 10 minutes (n = 2-3).Homogenates were subjected to immunoblot analysis, membranes were probed with antibodies against pHSLS563 and quantification was made using Image Lab Software (Bio-Rad Laboratories). Data are presented as fold of isoproterenol (ISO). Mean ± SD were used and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. N/A; not applicable.

HSLS563 in primary rat adipocytes						
			+ISO			
n = 2[#]-3	CTRL	ISO	3 I-BA[#]	10 I-BA	3 I-VA[#]	10 I-VA
Mean	0.02***	1	0.77	0.5*	0.66	0.51***
SD	0.12	N/A	0.35	0.25	0.52	0.03
			+ ISO			
n = 3	CTRL	ISO	3 PA	10 PA	3 BA	10 BA
Mean	0.09***	1	0.62**	0.26**	0.58	0.24**
SD	0.04	N/A	0.15	0.19	0.42	0.16
HSLS563 in human adipocytes						
			+ISO			
n = 3	CTRL	ISO	10 I-BA		10 I-VA	
Mean	0.20**	1	0.61**		0.31**	
SD	0.20	N/A	0.10		0.24	

BSCFAs and SCFAs inhibit insulin-induced phosphorylation of protein kinase B

To elucidate whether BSCFAs and SCFAs mediate their effect via PKB, a kinase involved in most acute insulin-mediated metabolic responses, we investigated their

Figure 2. BSCFAs inhibit lipolysis independent of PDE3B and PI3K in primary rat adipocytes. Lipolysis was measured after 1 hour of stimulation without (BASAL) or with 30 nM isoproterenol (ISO), in the absence (CTRL) or presence of 10 mM of isobutyric acid (I-BA), isovaleric acid (I-VA), acetic acid (AA) propionic acid (PA), butyric acid (BA) or insulin (INS) (1nM). The inhibitor for PDE3 (OPC3911) and the inhibitor for PI3K (Wortmannin;WM) were used in BASAL or ISO-stimulated lipolysis in the presence of the branched or non-branced SCFAs. A) I-BA, I-VA and INS in combination with OPC3911; B) I-BA, I-VA and INS in combination with WM; C) AA, PA, BA and INS in combination with WM. The values for I-BA, I-VA, AA, PA, BA and INS are related to CTRL (control without branched or non-branched SCFAs) in BASAL condition. For A-C, mean ± SD (n = 3) were used and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

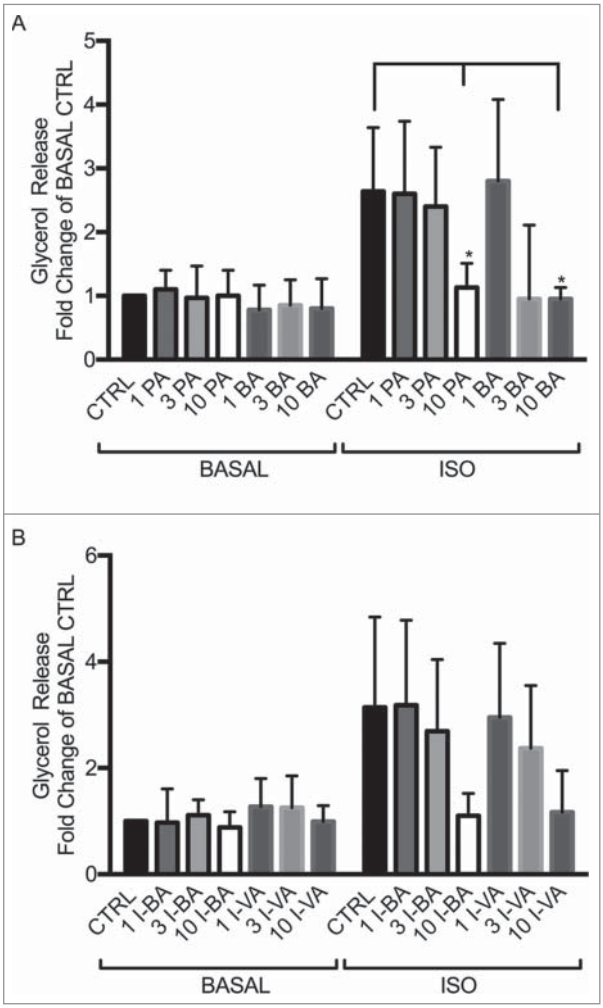

Figure 3. SCFAs and BSCFAs inhibit ISO-potentiated lipolysis in human adipocytes. Lipolysis was measured after 1 hour of stimulation without (BASAL) and with 30 nM isoproterenol (ISO) in the absence (CTRL) or presence of 3 and 10 mM of propionic acid (PA), butyric acid (BA), isobutyric acid (I-BA) or isovaleric acid (I-VA), as shown in A) for PA and BA and B) for I-BA and I-VA. The values are related to BASAL CTRL (condition without lipolytic agent and SCFA or BSCFAs). Mean ± SD (n = 3-4) were used and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

effect on PKB phosphorylation. The two regulatory sites, Thr^{308} and Ser^{473} in PKBα, are phosphorylated by PDK1 and the TORC2 complex, respectively, and are necessary for full activation of the kinase.[41] Figure 4 and Table 5 depict that insulin-mediated phosphorylation of PKB at Thr^{308} and Ser^{473} was decreased in response to BSCFAs and SCFAs in rat and human adipocytes.

Materials and methods

Animal model

Sprague Dawley male rats, 36-41 d of age, purchased from Charles River Laboratories (Germany), were kept under standardized conditions in the animal house facilities at Lund University. The Committee of ethical animal research in Malmö and Lund has approved all experimental procedures (permission number: M245-12).

Table 3. BSCFAs and acetic acid inhibit lipogenesis in primary rat adipocytes. Lipogenesis was measured after 30 minutes of stimulation without (BASAL) or with 1 nM insulin (INS) in the absence (CTRL) or presence of 1, 3 and 10 mM isobutyric acid (I-BA), isovaleric acid (I-VA) or acetic acid (AA). The values for I-BA, I-VA and acetic acid are related to respective CTRL (set to 1), either in a basal or an insulin-stimulated state. Mean ± SD (n = 3-4) were used and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. [a]11.2-fold, [b]6.4-fold, [c]5.7-fold increase in insulin-stimulated lipogenesis compared to BASAL.

		1 I-BA		3 I-BA		10 I-BA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	0.90	0.23	0.90**	0.23	0.58***	0.12
ISO	1[a]	0.98	0.09	0.88	0.14	0.61*	0.3
		1 I-VA		3 I-VA		10 I-VA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	0.89	0.08	0.95	0.03	0.88	0.14
ISO	1[b]	1.01	0.02	1.00	0.02	0.61*	0.11
		1 AA		3 AA		10 AA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	0.69	0.26	0.64**	0.07	0.22**	0.12
ISO	1[c]	0.84	0.11	0.62	0.21	0.05***	4E-4

Table 4. Isobutyric acid potentiates insulin-stimulated glucose uptake in primary rat adipocytes. Glucose uptake was measured after 30 minutes of stimulation without (BASAL) or with 1 nM insulin (INS) in the absence (CTRL) or presence of 1, 3 and 10 mM isobutyric acid (I-BA), isovaleric acid (I-VA) and acetic acid (AA). The values for I-BA, I-VA or AA are related to respective CTRL (set to 1), either in a basal or an insulin-stimulated state. Mean ± SD (n = 6-7) were used and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. [a]2.9-fold, [b]2.8-fold, [c]2.1-fold increase in insulin-stimulated glucose uptake compared to BASAL.

		1 I-BA		3 I-BA		10 I-BA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	1.29*	0.19	1.3**	0.18	1.24*	0.21
INS	1[a]	1.24***	0.05	1.27***	0.1	1.25**	0.11
		1 I-VA		3 I-VA		10 I-VA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	0.94	0.12	0.95	0.05	0.69**	0.1
INS	1[b]	1.02	0.03	0.74	0.2	1.01	0.12
		1 AA		3 AA		10 AA	
	CTRL Mean	Mean	SD	Mean	SD	Mean	SD
BASAL	1	0.98	0.09	0.87	0.14	0.82**	0.1
INS	1[c]	0.98	0.06	1.06	0.11	1.14	0.21

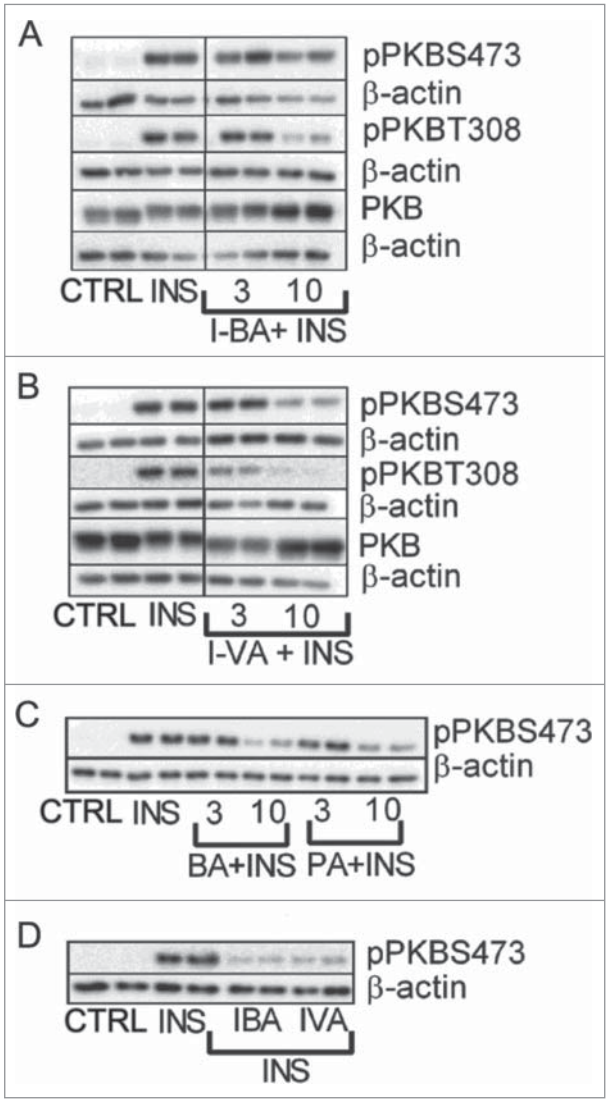

Figure 4. BSCFAs and SCFAs decrease phosphorylation of PKB in primary rat and human adipocytes. Primary rat or human adipocytes were stimulated with insulin (INS) in the absence (CTRL) or presence of 3 and 10 mM of SCFA or BCFA for 10 minutes. Homogenates were subjected to immunoblot analysis and membranes were probed with antibodies against PKB, PKB at serine 473 (S473) and PKB at threonine 308 (T308) as well as β-actin. For primary rat adipocytes, results (n = 3-6) are shown for isobutyric acid (I-BA) and isovaleric (I-VA) (A, B). For human adipocytes, results (n = 3–4) are shown for BA, PA, I- BA and I-VA (C, D). Representative blots are shown.

Table 5. BSCFAs and SCFAs decrease phosphorylation of PKB in primary rat and human adipocytes. Rat adipocytes were stimulated without (CTRL) or with INS in the absence or presence of 3 and 10 mM isobutyric acid (I-BA) and isovaleric acid (I-VA) for 10 minutes (n = 3). Human adipocytes were stimulated without (CTRL) or with INS in the absence or presence of 10 mM I-BA or I-VA for 10 minutes (n = 3) or 3 mM and 10 mM propionic acid (PA) or butyric acid (BA) for 10 minutes (n = 2-3). Homogenates were subjected to immunoblot analysis and membranes were probed with antibodies against PKB and PKB at serine 473 (S473) as well as β-actin and quantification was made using Image Lab Software (Bio-Rad Laboratories). Data are presented as fold of isoproterenol (ISO).Mean ± SD were used and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

PKBS473 in primary rat adipocytes[1]

			+ INS	
n = 3	CTRL	INS	10 I-BA	10 I-VA
Mean	0.09***	1	0.45**	0.46**
SD	0.07	0	0.14	0.14

PKBS473 in human adipocytes[2]

			+ INS	
n = 3	CTRL	INS	10 I-BA	10 I-VA
Mean	0.04***	1	0.60**	0.65*
SD	0.04	0	0.10	0.22

PKBS473 in human adipocytes[2]

			+ INS			
n = 2#-3	CTRL	INS	3 BA#	10 BA	3 PA#	10 PA
Mean	0.02***	1	0.84	0.30***	0.98	0.35***
SD	0.12	0	0.13	0.14	0.27	0.05

Human adipose tissue

Patients with scheduled reconstructive breast surgery, recruited by the surgeons at Skåne University Hospital, Sweden, were included in the study. Adipose tissue, removed from the abdominal subcutaneous region of the patients, reached the laboratory within one hour after surgery. The preparation of adipocytes was immediately initiated and usually 3 g of subcutaneous adipose tissue was used for an experiment. All experiments were approved by the Regional Ethics Committee in Lund, Sweden (Dnr 2013/298).

Adipocyte isolation

Epididymal white adipose tissue was isolated and digested in collagenase (Sigma Aldrich/C6885) for 30 minutes at 37°C with agitation and then washed as previously described.[42] The total volume of packed cells (lipocrit) in the adipocyte suspension was determined as described by Fine et al.[43] The adipocytes were resuspended and diluted to the final cell concentration (1-10%), depending on experimental setup.

Lipolysis

Lipolysis was measured as described by Dole et al.[44] Aliquots (400 μl) of 5% (rat) or 10% (human) suspensions of adipocytes were prepared in Krebs Ringers HEPES (KRH) containing 25 mM HEPES (pH 7.4),

120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1% BSA, 2 mM glucose, and 200 nM adenosine and incubated with or without 30 nM DL-Isoproterenol hydrochloride (ISO) (Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of 1, 3 and 10 mM isobutyric acid (Sigma-Aldrich), isovaleric acid (Merck, Kenilworth, NJ, USA) or acetic acid (Merck) at 37°C for 30 minutes in a shaking device (150 rpm). The cell-free medium was transferred to wells and buffer containing 50 mM glycine, 0.05% hydrazine-hydrate, 1 mM $MgCl_2$, 0.75 mg/ml ATP (Sigma-Aldrich), 0.375 mg/ml NAD (Roche, Pleasanton, CA, USA), 25 μg/ml glycerol-3-phosphate dehydrogenase (Roche) and 0.5 μg/ml glycerol kinase (Roche) was added. Lipolysis was measured at optical density of 340 as the release of glycerol into the cell-free medium.

De novo *lipogenesis from radio labeled glucose*

Lipogenesis was measured as described by Moody et al.[45] Aliquots (700μl) of a 2% suspension of adipocytes were prepared in KRH with low glucose (containing 0.55 mM glucose and 3.5% BSA without adenosine) and 14 μl D-[6−^{3}H (N)] glucose (22 μCi/ml) 0.81 Bq/ml (Perkin Elmer, Waltham, MA, USA) was added as substrate for lipogenesis. Cells were stimulated with 1 nM insulin (Novonordisk, Bagsvaerd, Denmark) in the presence or absence of 1, 3 and 10 mM isobutyric acid, isovaleric acid or acetic acid in a shaking device (100 rpm) at 37°C for 30 minutes. Incubations were stopped by addition of toluene-based scintillation liquid (VWR Chemicals, Radnor, PA, USA) containing 0.3 mg/ml 2.5-diphenyloxazole (PPO) (Sigma-Aldrich) and 5 mg/ml 1.4-bis-[4-methyl-5-phenyl-2-oxazolyl] benzene (POPOP) (Sigma-Aldrich) and the incorporation of ^{3}H into cellular lipids in the non-water soluble phase was determined by scintillation counting.

Glucose uptake

Glucose uptake was measured as described by Foley *et al.*[46] Aliquots (200 μl) of a 5% suspension of adipocytes were prepared in a glucose-free KRBH buffer containing 120 mM NaCl, 4 mM KH_2OP_4, 1 mM $MgSO_4$, 0.75 mM $CaCl_2$, 10 mM $NaHCO_3$, 1% BSA and 30 mM HEPES (pH 7.4). Radiolabeled glucose [100 μl D-[^{14}C (U)] (57.8 mCi/mmol) (2.1 GBq/mmol)] was added as substrate for glucose uptake. The adipocyte suspensions were incubated with or without 1 nM insulin in the presence or absence of 1, 3 and 10 mM isobutyric acid, isovaleric acid or acetic acid in a shaking device (100 rpm) at 37°C for 30 minutes. Adipocytes were separated from the medium by addition of 60 μl dinonylphthalate and then briefly centrifuged. Ultima Gold scintillation liquid (PerkinElmer, Waltham, MA, USA) was added to the cell suspensions and uptake of [^{14}C] glucose was determined by scintillation counting.

SDS-PAGE and Immunoblot Analysis

Aliquots of a 10% suspension of adipocytes were used for 10 minute incubations with 1 nM insulin in the presence or absence of different concentrations of isobutyric acid, isovaleric acid, acetic acid, propionic acid and butyric acid. Cells were homogenized with a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1mM EGTA, 1 mM EDTA, 1% NP40, 1 mM Na- orthovanadate, 50 mM NaF, 5 mM Na-pyrophosphate, 0.27 M sucrose, Nonidet P 40 (Sigma- Aldrich) and Complete Protease Inhibitor (containing inhibitors for serine-, cysteine-, and metalloproteases as well as calpains) (Roche) and centrifuged at 13 600 × *g* for 15 minutes. The total protein amount was determined according to Bradford. Samples (15-30 μg) were supplemented with NuPAGE sample reducing agent (Novex, Grand Island, NY, USA) and subjected to SDS-PAGE. Proteins were electrotransferred to Amersham Hybond-C Extra nitrocellulose membranes (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and the membranes were stained with Ponceau S (0.1% in 5% acetic acid) and then blocked with 10% milk in a buffer, consisting of 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% (v/w) Tween-20 for 60 min. Membranes with proteins were probed with antibodies for protein kinase B (PKB) (Cell Signaling Technology Inc., Danvers, MA, USA) , phospho-PKB (S473) (Cell Signaling) and phospho-PKB (T308) (Cell Signaling), phospho-HSL (S563) (Cell Signaling) and β-actin (Sigma Aldrich) and incubated overnight at 4°C. Proteins were detected using the chemiluminescent Super Signal West Pico Luminol/Enhancer solution (Thermo Fisher Scientific, Waltham, MA, USA) and the ChemiDoc XRS+ Imager (Bio-Rad Laboratories, Hercules, CA, USA.). Image Lab Software (Bio-Rad Laboratories) was used for quantification.

Statistics

Data are presented as mean ± SD from the indicated number of experiments. Statistically significant differences were analyzed using Student's t-test and significance levels were accepted when $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Discussion

The ability of colon-produced SCFAs and BSCFAs to act upon peripheral tissues like adipose tissue requires that a

fraction of these molecules reaches the systemic circulation. Beyond the liver, the concentration of all 3 SCFAs (acetic acid > propionic acid > butyric acid) as well as isobutyric acid have been estimated to be in the micromolar range in the systemic circulation.[4,21,47-50] Furthermore, using stable isotope technology, systemic availability of colonic-administered acetate, propionate and butyrate was shown to be 36%, 9% and 2%, respectively.[51] In another study, a net uptake of the SCFAs by adipose tissue, measured as the arteriovenous difference across the tissue times the tissue could be demonstrated.[50] In summary, SCFAs and BSCFAs are available in the systemic circulation albeit at lower concentration than used in the current study having isolated primary adipocytes as *in vitro* model.

The mechanisms whereby BSCFAs and SCFAs mediate their effects observed on biological responses and cellular signaling that have been reported herein, are still unclear. However, it has become increasingly evident that all macronutrients, carbohydrates, proteins and lipids, also play an important role in the regulation of energy metabolism as signaling molecules.[52] Moreover, free fatty acid receptors (FFARs) are likely to play important roles in fatty acid- mediated regulation of energy metabolism.[10] Specifically, FFAR2 and 3 have been linked to a number of effects exerted by SCFAs in adipocytes, for example, FFAR2 was shown to suppress lipolysis and stimulate adipogenesis as well as adipocyte differentiation whereas FFAR3 was shown to induce leptin expression.[34-36,53] With regards to BSCFAs and their binding to FFARs, to our knowledge, less is known. In addition to acting as signaling molecules, BSCFAs and SCFAs could mediate metabolic effects in adipocytes by entering various metabolic routes as discussed below.

The finding that isobutyric and isovaleric acid inhibited isoproterenol-mediated lipolysis and decreased isoproterenol-induced phosphorylation of HSL, indicate a role for BSCFAs in the regulation of energy homeostasis by protecting against lipotoxicity, as has previously been reported for SCFAs.[33-35] Importantly, the mechanism behind the anti-lipolytic effect of BSCFAs and SCFAs appears not to involve components utilized by insulin to mediate inhibition of lipolysis. Namely, BSCFAs and SCFAs induced inhibition of lipolysis in the presence of inhibitors for PI3 kinase and PDE3B, whereas the anti-lipolytic effect of insulin was inhibited as expected under those conditions. Furthermore, phosphorylation of PKB, a key kinase in mediating acute metabolic effects of insulin such as inhibition of lipolysis, was downregulated both at Ser^{473} and Thr^{308}, 2 activity controlling phosphorylation sites.[41] Having that in mind, it will be interesting to test the ability of BSCFAs and SCFAs to inhibit lipolysis in insulin-resistant adipocytes isolated from obese individuals. Obese adipose tissue often has dysregulated lipolysis, leading to excessive release of fatty acids and decreased insulin sensitivity.[29-31] As shown in this paper, both BSCFAs and SCFAs reduce phosphorylation of HSL whereas significant inhibition of lipolysis was only obtained with SCFAs.

The finding that BSCFAs decrease the rate of *de novo* lipogenesis,[54] measured as the incorporation of [^{3}H]-labeled glucose into cellular lipids, is in agreement with previous results with non-branched SCFAs.[33] Altogether, these findings indicate that BSCFAs and SCFAs facilitate storage of diet-derived lipids in adipocytes rather than performing *de novo* synthesis, which at least under conditions of fat overconsumption appears metabolically beneficial. Also, the effect to diminish *de novo* lipogenesis might lead to an increased β-oxidation due to reduced level of malonyl CoA, a metabolite known to inhibit the transport of fatty acids into the mitochondria.[55] The inhibitory effect on PKB phosphorylation observed in response to the branched and non-branched SCFAs and the phosphorylation and inactivation of ACC[33] might be molecular mechanisms for diminished *de novo* lipogenesis. Isovaleric acid-containing porpoise oil, given in the diet, has ameliorating effects on fatty liver in OLETF (Otsuka Long- Evans Tokushima Fatty) rat, a model for type 2 diabetes, by increasing serum levels of adiponectin and enhancing lipoprotein synthesis and secretion.[56] However, isobutyric and isovaleric acid have been scarcely investigated with regard to effects on *de novo* lipogenesis.

The finding that isobutyric acid significantly increased both basal and insulin-stimulated glucose uptake is in agreement with recently obtained results for propionic acid and to some extent also for butyric acid in rodent primary and differentiated adipocytes.[33] Phosphorylation of PKB is one key event in insulin-induced glucose uptake,[41] however, phosphorylation of PKB was shown to be downregulated by both branched and non-branched SCFAs. In agreement with our findings on PKB, Kimura et al have observed a decreased insulin- mediated phosphorylation of PKB at Ser^{473} in response to acetic acid in adipocytes, an effect that was mediated by GPR43.[36]

In addition to acting as signaling molecules, BSCFAs and SCFAs could act as energy substrates in adipocytes by entering routes in lipid and carbohydrate metabolism as has been described in colonocytes and hepatocytes.[6,9,21,57] In hepatocytes, acetate and butyrate have been described to be mostly introduced into lipid biosynthesis, whereas propionate will mainly be incorporated into gluconeogenesis.[6] With regards to adipocytes, BSCFAs and SCFAs might enter the lipogenic route after uptake and activation by short chain CoA synthases.[58-60] Thus, the inhibitory effect we observe on *de novo* lipogenesis from glucose could, at least to some extent, be explained by the ability of BSCFAs/SCFAs to generate

"competing" non-labeled acetyl-CoAs for the synthesis of fatty acids. Likewise, it is possible that SCFAs and BSCFAs, by the generation of long chain acyl-CoAs, inhibit lipolysis by the inhibition of ATGL (triglyceride lipase) and/or HSL.[61-63]

To summarize, in the current investigation we have observed a number of effects in response to BSCFAs and SCFAs on lipid and glucose metabolism and signaling in adipocytes, possible mediated by FFAR 2 and 3. However, the possibility that the effects observed are mediated by BSCFAs and SCFAs acting as substrates by entering various routes in lipid and carbohydrate metabolism in adipocytes should not be excluded.

Abbreviations

SCFA	short-chain fatty acid
BSCFA	branched short-chain fatty acids
BA	butyric acid
PA	propionic acid
I-BA	isobutyric acid
I-VA	isovaleric acid
PDE3B	phosphodiesterase 3B
HSL	hormone sensitive lipase
PKB	protein kinase B
PI3 kinase	phosphatidyl inositol 3-kinase

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

All authors contributed to the design of the study and manuscript writing. EH contributed to the conduct of the study and data collection, whereas all authors contributed with analysis as well as data interpretation.

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