Am J Physiol Cell Physiol 319: C1151–C1157, 2020.
First published October 7, 2020; doi:10.1152/ajpcell.00377.2020.

RESEARCH ARTICLE

Exploring mechanistic links between extracellular branched-chain amino acids and muscle insulin resistance: an in vitro approach

Hannah Crossland, Kenneth Smith, Iskandar Idris, Bethan E. Phillips, Philip J. Atherton,* and Daniel J. Wilkinson*

Medical Research Council-Versus Arthritis Centre for Musculoskeletal Ageing Research, National Institute for Health Research Nottingham Biomedical Research Centre, Clinical, Metabolic and Molecular Physiology, University of Nottingham, Royal Derby Hospital, Derby, United Kingdom

Submitted 28 July 2020; accepted in final form 30 September 2020

Crossland H, Smith K, Idris I, Phillips BE, Atherton PJ, Wilkinson DJ. Exploring mechanistic links between extracellular branched-chain amino acids and muscle insulin resistance: an in vitro approach. *Am J Physiol Cell Physiol* 319: C1151–C1157, 2020. First published October 7, 2020; doi:10.1152/ajpcell.00377.2020.—Branched-chain amino acids (BCAAs) are essential for critical metabolic processes; however, recent studies have associated elevated plasma BCAA levels with increased risk of insulin resistance. Using skeletal muscle cells, we aimed to determine whether continued exposure of high extracellular BCAA would result in impaired insulin signaling and whether the compound sodium phenylbutyrate (PB), which induces BCAA metabolism, would lower extracellular BCAA, thereby alleviating their potentially inhibitory effects on insulin-mediated signaling. Prolonged exposure of elevated BCAA to cells resulted in impaired insulin receptor substrate 1/AKT signaling and insulin-stimulated glycogen synthesis. PB significantly reduced media BCAA and branched-chain keto acid concentrations and increased phosphorylation of AKT [+2.0 ± 0.1-fold; $P < 0.001$ versus without (−)PB] and AS160 (+3.2 ± 0.2-fold; $P < 0.001$ versus −PB); however, insulin-stimulated glycogen synthesis was further reduced upon PB treatment. Continued exposure of high BCAA resulted in impaired intracellular insulin signaling and glycogen synthesis, and while forcing BCAA catabolism using PB resulted in increases in proteins important for regulating glucose uptake, PB did not prevent the impairments in glycogen synthesis with BCAA exposure.

branched-chain amino acids; in vitro; insulin resistance; phenylbutyrate; skeletal muscle

INTRODUCTION

The branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) are essential amino acids that play critical roles in physiological processes ranging from protein synthesis and modulation of glucose metabolism to regulation of appetite and adiposity (11, 18, 29). Recent studies, however, have identified associations between levels of plasma BCAAs and risk of insulin resistance and type 2 diabetes mellitus (T2DM), although it is not clear whether BCAAs can cause insulin resistance or whether increased circulating BCAAs may reflect a consequence of reduced insulin action (9, 21). More detailed investigations into the mechanistic roles that BCAAs and their intermediary metabolites have in muscle glucose metabolism, and whether sustained elevations of circulating BCAA can cause insulin resistance, are therefore necessary.

In terms of the potential underlying mechanisms by which BCAAs have been linked with insulin resistance, Hernández-Alvarez et al. (10) observed reduced skeletal muscle expression of genes involved in BCAA metabolism in T2DM patients, while insulin-resistant rats were reported to have an impaired capacity for BCAA catabolism in skeletal muscle (6). Mice heterozygous for the BCAA-related enzyme methylmalonyl-CoA mutase (thereby resulting in dysregulated BCAA metabolism) displayed increases in muscle triglyceride accumulation and increased body weight following high-fat feeding (17). Specifically, it has been proposed that the valine catabolite 3-hydroxyisobutyrate (3HIB) can promote insulin resistance by promoting lipid accumulation in vivo (14), and cultured myotubes treated with 3HIB exhibited suppressed insulin signaling and mitochondrial metabolism. Elevated plasma 3HIB has also been associated with future risk of developing T2DM (19). The mechanistic target of rapamycin (mTOR) pathway has also been proposed as a further mechanism by which BCAAs may cause insulin resistance, through inhibition of insulin receptor substrate 1 (IRS-1) via serine phosphorylation by p70 S6K1 (30). Collectively, these findings support a potential causal role of BCAAs to suppress insulin sensitivity either directly, or via the products of their catabolism.

Recent studies have focused on strategies aimed at lowering BCAA as a means of improving insulin resistance. Cummings et al. (5) observed that reducing dietary BCAA rapidly led to fat loss and increased glucose tolerance in obese mice, while BCAA restriction in obese rats improved skeletal muscle insulin sensitivity and fatty acid metabolism in obese rats (25). In obese (*ob*/*ob*) mice, increasing BCAA catabolic flux using an inhibitor of BCKA dehydrogenase kinase resulted in reduced levels of BCAA and decreased insulin resistance (31). However, investigations into the strategy of BCAA restriction or depletion in humans for improving metabolic health have been lacking. In patients with Maple Syrup Urine Disease, a disorder of BCAA metabolism that results in elevations of BCAAs in plasma, the compound sodium phenylbutyrate (PB) was found to effectively reduce plasma levels of BCAAs and branched-chain alpha-keto acids (BCKAs) by increasing flux through the BCKA dehydrogenase complex (4). PB, which is converted into the active metabolite phenylacetate in vivo, combines with glutamate to form phenylacetylglutamine, which is then disposed of in the urine (4). In a study of overweight and obese males, PB administration was demonstrated to partially improve lipid-induced

* P. J. Atherton and D. J. Wilkinson contributed equally to this work as joint senior authors.
Correspondence: D. J. Wilkinson (D.Wilkinson@nottingham.ac.uk).

insulin resistance, although circulating BCAA concentrations were not measured in this study (26). These findings suggest that treatment with PB could provide benefits for metabolic disorders such as insulin resistance in humans, potentially through the promotion of BCAA intermediary metabolism (moreover, a therapeutic approach may be better tolerated and have greater compliance compared with a dietary approach).

Based on the combined evidence to date, the aims of the present study were to investigate the mechanistic links between BCAA, intracellular insulin signaling, and muscle glucose metabolism. Using an in vitro skeletal muscle cell model, we hypothesized that lowering extracellular BCAA concentrations using the compound PB would effectively reduce molecular markers of insulin resistance under conditions of sustained increases in media BCAAs by accelerating their catabolism and reducing concentrations of BCAAs and their downstream metabolites thereby alleviating their inhibitory effects on insulin signaling and substrate metabolism.

METHODS

C2C12 cell culture experiments. Murine C2C12 myoblasts (passage 6–8; ECACC, Salisbury, UK) were cultured at 37°C and 5% CO_2 in minimum essential medium alpha (MEMα; Thermo Fisher Scientific) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) antibiotic-antimycotic solution (all from Sigma-Aldrich, UK). Cells were seeded onto six-well multidishes (Nunclon Delta; Thermo Fisher Scientific), and once cells reached ~90% confluency, differentiation was induced by changing the medium to MEMα containing 2% (vol/vol) horse serum. A media change was carried out every 48 h, and experiments were performed on *days 4–5* postinduction of differentiation.

For measuring the effects of PB under basal and insulin-stimulated conditions, and with normal (1×) or elevated (4×) media BCAA, 4 days after induction of differentiation, myotubes were treated for 48 h with normal (1×) or elevated (4×) media BCAA concentrations. In the MEMα used, unsupplemented BCAA media concentrations were all ~0.4 mM, according to the manufacturer. For basal experiments, cells were treated with or without 7.5 mM PB for 6 h (in the range of previously used doses, e.g., Ref. 3, 12) after the 48-h period. For insulin-stimulated experiments, cells were treated with or without 7.5 mM PB and then after 3 h, the media were replaced with fresh media supplemented with insulin (to a final concentration of 300 nM) and cells were collected after a further 3 h ($n = 6$ replicates for each treatment group). Where media were replaced, 1× and 4× BCAA concentrations were maintained in the respective treatment groups, as well as PB treatments (such that cells were exposed to 6-h PB in both basal and insulin-stimulated experiments). Media were collected and cells were harvested in homogenization buffer (50 mM Tris·HCl, pH7.5, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM NaF, and complete protease inhibitor cocktail tablet; Roche, UK) for immunoblotting analysis and measurement of intracellular glycogen concentrations (see below).

Measurement of media amino acids and branched-chain alpha-keto acids. Cell culture media (100 μL) with 10-μL internal standard (a mix of stable isotopically labeled amino acids) added were purified by passing through H^+ dowex resin columns and were eluted into 2 mol/L NH_4OH and dried down. Amino acids were derivatized to their *tert*-butyldimethylsilyl (tBDMS) esters, and concentrations were determined using gas chromatography-mass spectrometry (GC-MS; Trace 1300-ISQ, Thermo Scientific) against a standard curve of known concentrations.

Media concentrations of the BCKA [alpha-ketoisocaproic acid (KIC); alpha-keto-beta-methylvaleric acid (KMV), and alpha-ketoisovaleric acid (KIV)] were measured by mixing 100 μL of cell culture media with alpha-ketovaleric acid as internal standard, deproteinising using ice-cold ethanol and drying down under nitrogen. The BCKAs were derivatized to quinoxalinol-t-BDMS derivatives, and concentrations were determined using GC-MS (Trace 1300-ISQ, Thermo Scientific) with a standard curve of known concentrations of each BCKA.

Western blot analysis. Samples were lysed by repeatedly passing through gel-loading pipette tips, and lysates were centrifuged at 13,000 g for 10 min. Ten micrograms of protein were loaded onto Criterion XT 12% Bis-Tris gels (Bio-Rad, UK), and samples were electrophoresed at 200 V for 1 h. Sample transfer to PDVF membranes was performed at 100 V for 45 min, and membranes were blocked in 5% (wt/vol) milk for 1 h at room temperature. Primary antibody incubation was carried out overnight at 4°C with the following antibodies: AKT Ser473 (no. 4060), AS160 Thr642 (no. 8881), and IRS-1 Ser1101 (no. 2385; Cell Signaling Technology, UK). After antibody incubations, membranes were washed with 1× TBS-Tween and incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (no. 7074; Cell Signaling Technology, UK; 1:2,000 dilution) for 1 h at room temperature. After further washing with TBS-Tween, bands were detected using Chemiluminescent HRP substrate (Millipore EMD) with a Chemidoc XRS imaging system (Bio-Rad, UK). Staining of the membrane with Coomassie brilliant blue was used for total protein normalization.

Measurement of cellular glycogen. Cellular glycogen concentrations were determined in samples from the insulin stimulation experiments using the colorimetric Glycogen Assay Kit II (Abcam), according to the manufacturer instructions. Glycogen levels were normalized to total protein content of each sample.

Statistical analyses. Data were analyzed using two-way ANOVA (using Tukey's multiple comparison post hoc test) to evaluate differences between treatment groups. $P < 0.05$ was considered as statistically significant. All data are presented as means ± SE.

RESULTS

Effects of elevated BCAA on media amino acid and alpha-keto acid concentrations in C2C12 myotubes. For initial experiments, we sought to assess how sustained elevations in media BCAA concentrations would affect glycogen synthesis under basal and insulin-stimulated conditions, first measuring the impact of sustained high BCAA on concentrations of other amino acids and alpha-keto acids in the media. Under normal BCAA (1×) conditions (i.e., standard in the supplied media), insulin/feeding resulted in increases in media BCAA concentrations as well as glutamine and glutamate (Table 1), indicative of the replenishment in substrates postmedia change (compared with the basal condition, which was 48 h postmedia change). Elevating media BCAA (4×) did not have any overall effects on concentrations of media glutamine or glutamate; however, elevated media BCAA caused a reduction in alanine concentrations ($P < 0.001$ versus 1× BCAA for basal conditions, $P < 0.05$ versus 1× BCAA for insulin-stimulated conditions; Table 1). Concentrations of media BCKA were significantly increased under conditions of high BCAA ($P < 0.05$ main effect by ANOVA; Table 2).

Effects of elevated BCAA on glycogen content and insulin signaling in C2C12 myotubes. Cells were analyzed for glycogen content as well as phosphorylation of proteins important in mediating insulin signaling pathways to assess the impact of BCAA exposure levels (i.e., 1× and 4×) upon insulin-signaling and glycogen content. Under basal conditions, 4× BCAA resulted in decreased glycogen content (−5% versus 1× BCAA; $P < 0.05$; Fig. 1*A*), while insulin-stimulated increases in muscle glycogen content were suppressed under

Table 1. *Media amino acid concentrations in C2C12 myotubes with or without elevated media branched chain amino acids and insulin treatment*

	1× BCAA		4× BCAA	
	Basal	INS/feed	Basal	INS/feed
Val	277 ± 8	405 ± 37**	1,101 ± 24###	1,457 ± 79**###
Leu	262 ± 8	392 ± 15***	1,248 ± 29###	1,344 ± 64###
Ile	377 ± 7	476 ± 32*	1,468 ± 37###	1,499 ± 56###
BCAA (sum)	916 ± 23	1,275 ± 78**	3,818 ± 75###	4,334 ± 214###
Gln	1,505 ± 185	2,093 ± 174*	1,449 ± 57	1915 ± 108**
Glu	127 ± 8	507 ± 26***	119 ± 3	460 ± 33***
Ala	521 ± 38	382 ± 41*	173 ± 29###	250 ± 26#

Data are means ± SE and units are μmol/l. C2C12 myotubes were treated for 48 h with or without elevated (4×) media branched chain amino acids (BCAAs), and then cells were treated for 3 h with or without feeding plus insulin (INS; 300 mM; n = 5–6 per group). Media were analyzed for concentrations of selected amino acids. *$P < 0.05$ vs. basal, **$P < 0.01$ vs. basal, ***$P < 0.001$ vs. basal. #$P < 0.05$ vs. 1× BCAA, ###$P < 0.001$ vs. 1× BCAA.

high BCAA conditions (Fig. 1*A*). Overall, 4× BCAA conditions resulted in lower phosphorylation of AKT (−32% versus 1× BCAA under basal conditions; $P < 0.05$; −30% versus 1× BCAA under insulin-stimulated conditions; $P < 0.05$; Fig. 1*B*). Under conditions of 4× BCAA, AS160 phosphorylation was reduced under basal conditions (−44% versus 1× BCAA; $P < 0.01$), while under insulin-stimulated conditions, there was no difference in AS160 phosphorylation compared with 1× BCAA conditions (Fig. 1*C*). Under insulin-stimulated conditions, increased media BCAA resulted in increases in IRS-1 serine phosphorylation relative to normal BCAA conditions (Fig. 1*D*).

Effects of PB treatment on media amino acid and keto acid concentrations in C2C12 myotubes. To investigate whether accelerated BCAA catabolism using PB could restore the impairments in insulin signaling and glucose metabolism with high BCAA, the impact of PB treatment was assessed under basal conditions or following a feed supplemented with insulin, with prior 48-h treatment with high concentrations of media BCAA. Under high BCAA conditions (4×), there were significant reductions in media concentrations of valine with PB treatment under basal [1,101 ± 24 versus 973 ± 16 μmol/l, $P < 0.05$ versus without (−)PB] and insulin-stimulated (1,457 ± 79 versus 1,146 ± 14 μmol/l, $P < 0.001$ versus −PB) conditions, while leucine (1,377 ± 90 versus 1,105 ± 30 μmol/l, $P < 0.01$ versus −PB) and isoleucine (1,533 ± 85 versus 1,323 ± 36 μmol/l, $P < 0.05$ versus −PB) were reduced with PB treatment, under insulin-stimulated conditions only (Fig. 2, *A–C*). Under 4× BCAA conditions, PB did not significantly alter media concentrations of KIC, KIV, or KMV either with basal or insulin-stimulated/fed conditions (Fig. 2, *D–F*).

Effects of PB treatment on glycogen content and insulin signaling in C2C12 myotubes. Under conditions of elevated BCAA, there was a decrease in glycogen concentration following PB treatment under basal (0.070 ± 0.0008 versus 0.074 ± 0.0007 μg/μg protein; $P < 0.05$ versus −PB) and insulin-stimulated conditions (0.063 ± 0.0003 versus 0.074 ± 0.0013 μg/μg protein; $P < 0.001$ versus −PB; Fig. 3*A*). AKT phosphorylation was elevated with PB under high BCAA conditions, both in the basal (2.0 ± 0.1-fold versus −PB; $P < 0.001$) and insulin-stimulated (2.0 ± 0.1-fold versus −PB; $P < 0.001$) state (Fig. 3*B*), while AS160 Thr642 phosphorylation was also significantly increased by PB treatment (3.2 ± 0.2-fold versus −PB in basal conditions; $P < 0.001$; Fig. 3*C*). IRS-1 Ser1101 phosphorylation was not significantly altered by PB treatment under insulin-stimulated or basal conditions (Fig. 3*D*).

DISCUSSION

To date, the role of BCAA in mediating insulin resistance and metabolic dysregulation in T2DM remains unclear, since BCAAs are known to be important in a wide range of metabolic pathways and are critical regulators of protein turnover and fuel metabolism, but elevated circulating BCAAs have recently been significantly correlated with insulin resistance in healthy individuals and in metabolic disorders such as T2DM (1, 2, 8, 27). The aims of the present study therefore were to investigate the mechanistic links between BCAAs, intracellular insulin signaling, and muscle glucose metabolism, using an in vitro skeletal muscle model. We hypothesized that lowering extracellular BCAAs by accelerating their catabolism (using the compound PB), thereby reducing concentrations of BCAAs and their downstream metabolites, would alleviate their inhibitory effects on insulin signaling and substrate metabolism. We observed that exposing the cells to sustained increases in extracellular BCAA caused impairments in insulin-stimulated glycogen synthesis and intracellular insulin signaling. Interestingly, while murine C2C12 myotubes treated with PB had lowered media BCAA concentrations and increased molecular markers of proteins that regulate glucose uptake (potentially suggestive of improved insulin sensitivity), cells exhibited no improvements in insulin-mediated glycogen synthesis.

Impact of elevated BCAA on insulin signaling and glycogen content in C2C12 myotubes. The present study was motivated by recent observations that higher concentrations of BCAA were associated with insulin resistance and an increased risk of T2DM in adults (1, 8) and that targeting catabolism of BCAA may reduce levels of BCAA, and thereby decrease insulin resistance (31). In a group of subjects with T2DM, higher concentrations of BCAA were associated with homeostatic model assessment of insulin resistance (HOMA-IR) and an increased risk of T2DM (8). Some studies however have demonstrated that BCAA or leucine supplementation can improve insulin sensitivity in individuals with T2DM (23) and glucose tolerance in patients with chronic liver

Table 2. *Media alpha-keto acid concentrations in C2C12 myotubes with or without elevated media branched chain amino acids and insulin treatment*

	1× BCAA		4× BCAA	
	Basal	INS/feed	Basal	INS/feed
KIC	34 ± 1	24 ± 4*	210 ± 18###	67 ± 8***##
KMV	40 ± 1	17 ± 3***	166 ± 10###	61 ± 10***##
KIV	19 ± 1	11 ± 2**	79 ± 5###	27 ± 3***##

Data are means ± SE and units are μmol/l. C2C12 myotubes were treated for 48 h with or without elevated (4×) media branched chain amino acids (BCAAs), and then cells were treated for 3 h with or without feeding plus insulin (INS; 300 mM; n = 5 per group). Media were analyzed for concentrations of selected alpha-keto acids. KIC, alpha-ketoisocaproic acid (KIC); KMV, alpha-keto-beta-methylvaleric acid; KIV, alpha-ketoisovaleric acid. *$P < 0.05$ vs. basal, **$P < 0.01$ vs. basal, ***$P < 0.001$ vs. basal. ##$P < 0.01$ vs. 1× BCAA, ###$P < 0.001$ vs. 1× BCAA.

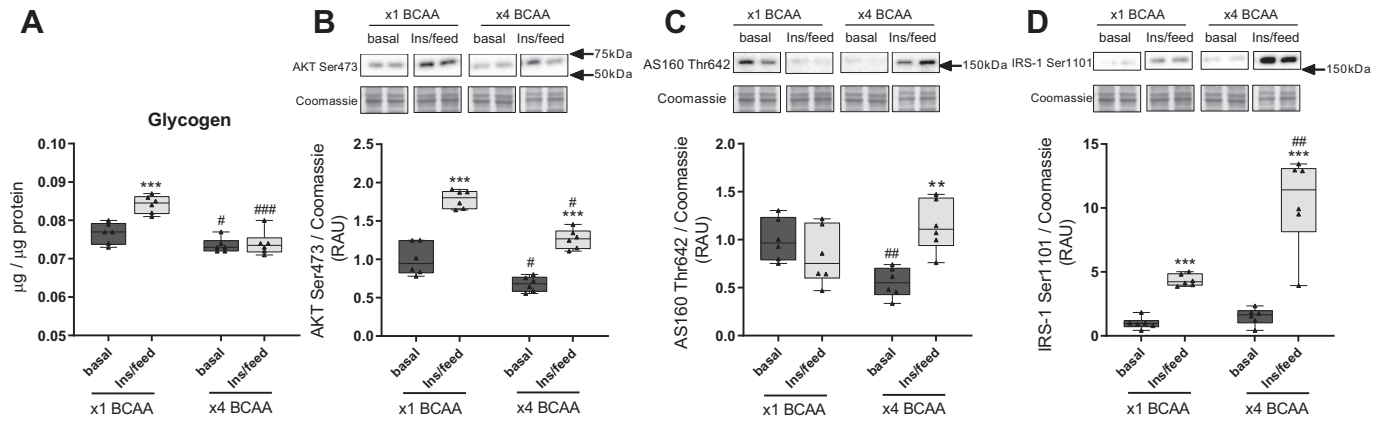

Fig. 1. Glycogen content and insulin-related signaling changes in C2C12 myotubes with or without elevated media branched chain amino acids (BCAAs) and insulin treatment. C2C12 myotubes were treated for 48 h with or without elevated (4×) media BCAA, and then cells were treated for 3 h with or without feeding/insulin (300 mM; $n = 6$ per group). Cells were analyzed for glycogen content (normalized against total protein; *A*) and phosphorylation of AKT (Ser473; *B*), AKT substrate of 160 kDa (AS160; Thr642; *C*), and insulin receptor substrate 1 (IRS-1; Ser1101; *D*). Triangles represent individual data points. $^{**}P < 0.01$ vs. basal, $^{***}P < 0.001$ vs. basal (by ANOVA). $\#P < 0.05$ vs. 1× BCAA, $\#\#P < 0.01$ vs. 1× BCAA, $\#\#\#P < 0.001$ vs. 1× BCAA (by ANOVA).

disease (15, 20). BCAA supplementation is also beneficial to energy expenditure, through brown adipose tissue utilizing BCAA in mitochondria, thus promoting BCAA clearance (28). These findings highlight the uncertainty that has surrounded the role of BCAA, and their metabolites, in the regulation of insulin resistance and metabolic dysfunction.

In this study, we were able to develop a model in vitro that allowed us to directly examine the impact of increased/decreased extracellular BCAA on the regulation of glycogen content in skeletal muscle. Exposure of myotubes for 48 h with elevated BCAA resulted in marked increases in levels of BCKA (KIV, KMV, and KIC) in the media. This could reflect a build-

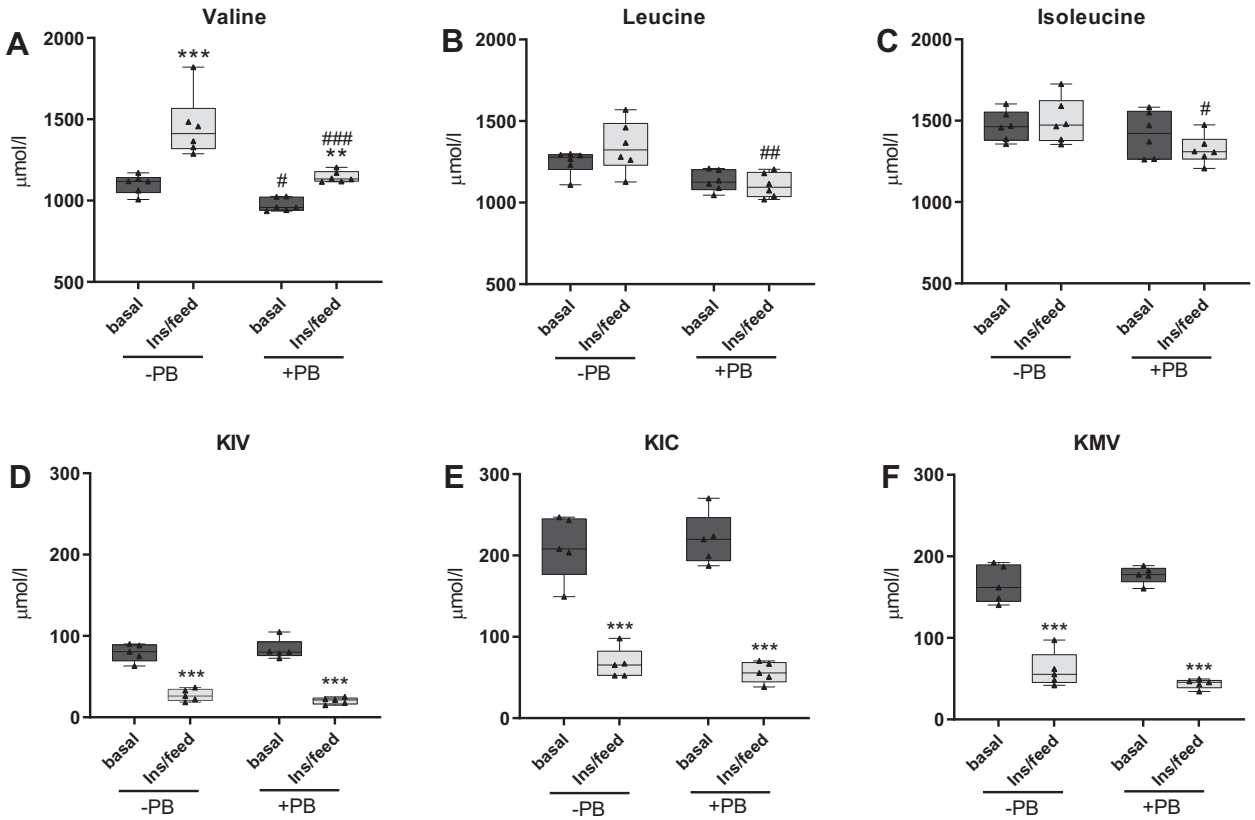

Fig. 2. Media amino acid (*A–C*) and alpha-keto acid (*D–F*) concentrations following phenylbutyrate (PB) treatment under conditions of elevated branched-chain amino acids (BCAAs). C2C12 myotubes were treated for 48 h with elevated (4×) media BCAA, and then cells were treated for 3 h with or without 7.5 mM PB and then a further 3 h with or without feeding plus insulin (300 mM; $n = 5$–6 per group). Media were analyzed for concentrations of selected amino acids and alpha-keto acids. KIC, alpha-ketoisocaproic acid (KIC); KMV, alpha-keto-beta-methylvaleric acid; KIV, alpha-ketoisovaleric acid. Triangles represent individual data points. $^{**}P < 0.01$ vs. basal, $^{***}P < 0.001$ vs. basal (by ANOVA). $\#P < 0.05$ vs. without (−)PB, $\#\#P < 0.01$ vs. −PB, $\#\#\#P < 0.001$ vs. −PB (by ANOVA).

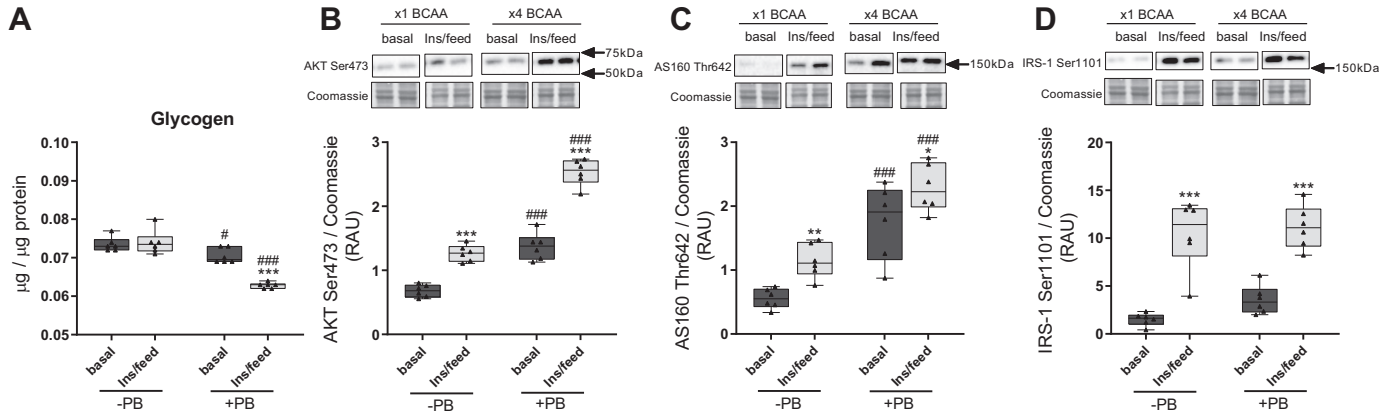

Fig. 3. Glycogen content and insulin-related signaling change following phenylbutyrate (PB) treatment under conditions of elevated branched-chain amino acids (BCAA). C2C12 myotubes were treated for 48 h with elevated (4×) media BCAA, and then cells were treated for 3 h with or without 7.5 mM PB and then a further 3 h with or without feeding plus insulin (300 mM; $n = 6$ per group). Cells were analyzed for glycogen content (normalized against total protein; *A*) and phosphorylation of AKT (Ser473; *B*), AKT substrate of 160 kDa (AS160; Thr642; *C*), and insulin receptor substrate 1 (IRS-1; Ser1101; *D*). Triangles represent individual data points. $*P < 0.05$ vs. basal, $**P < 0.01$ vs. basal, $***P < 0.001$ vs. basal (by ANOVA). $\#P < 0.05$ vs. without (−)PB, $\#\#\#P < 0.001$ vs. −PB (by ANOVA).

up of intermediates in the BCAA catabolism due to excess substrates or potentially that there were impairments in BCAA catabolism. This is in line with previous findings that insulin-resistant rats exhibited an impaired capacity for BCAA catabolism in skeletal muscle (6) and supports the suggestion that targeting catabolism of BCAA may reduce levels of BCAA and thereby decrease insulin resistance in these settings (31). How the elevated BCAA may have impacted on pyruvate utilization, and thereby ATP production and muscle mitochondrial metabolism, was not assessed in our study but remains a valuable area of future study. In these experiments, we sought to measure how sustained elevations in media BCAA concentrations would affect glycogen content under basal and insulin-stimulated conditions, and at the molecular level, we examined the effects of high BCAA on intracellular signaling. Under basal and fed conditions, AKT activation was decreased with 4× BCAA, suggesting an impairment in activity due to increased BCAA. Under basal conditions, AS160 phosphorylation appeared to be decreased with 4× BCAA, while this decrease was not observed under insulin-stimulated conditions, indicating that elevated BCAA had different impacts on the regulation of glucose uptake under basal and insulin-stimulated settings. It is unclear why AS160 phosphorylation was not induced by insulin under normal BCAA conditions but could feasibly reflect an early but transient stimulation by insulin that was restored to baseline values by 3 h postfeed. IRS-1 serine phosphorylation was increased following prolonged high BCAA administration under insulin-stimulated conditions, which together indicates an inhibitory impact on insulin-regulated pathways. These observations are in agreement with previous data proposing that BCAA may cause insulin resistance through inhibition of IRS-1, potentially downstream of mTOR activation (30). High levels of BCAA also appeared to inhibit insulin-stimulated increases in glycogen content, suggesting that, along with changes in intracellular insulin signaling, sustained increases in BCAA had negative impacts on insulin sensitivity at the level of glycogen synthesis. Overall, the model used in the present study allowed us to directly assess the impact of increased/decreased extracellular BCAA on the regulation of glucose utilization in skeletal muscle cells.

Impact of PB treatment on insulin signaling and glycogen content in C2C12 myotubes. Subsequent experiments addressed whether accelerating BCAA metabolism under conditions of elevated BCAA, using the compound PB, would lower extracellular BCAA, thereby alleviating their inhibitory effects on insulin-mediated signaling. Treatment with the compound PB [as expected from previous studies (12)] significantly reduced media concentrations of BCAA under conditions of elevated BCAA (under fed conditions). Under conditions of high BCAA, while PB was able to suppress BCAA concentrations, elevations in BCKA were not significantly altered, which could indicate some impairments in the BCAA catabolic pathways under conditions of elevated BCAA. Thus, in our present model of BCAA-induced muscle insulin resistance, the build-up of extracellular BCKA was not prevented by PB.

To explore the mechanistic links between BCAA catabolism and insulin signaling pathways, we measured selected molecular targets known to be important in regulating glucose homeostasis. PB treatment has previously been reported to accelerate glucose uptake and glycogen synthesis in muscle (13), demonstrating one mechanism by which PB could provide benefits in terms of improving insulin sensitivity. Treatment with PB increased AKT phosphorylation (i.e., activation) in the high BCAA condition, while with elevated BCAA, PB further increased AS160 activation, both of which are critical steps in the translocation of glucose transporter 4 (GLUT4) transporters to the cell surface and which would be predicted to increase glucose uptake (24). These findings are in agreement with previous studies reporting increased glucose uptake in response to PB administration (13), although in contrast to the work of Hu et al., our findings were indicative of increased glucose uptake into muscle cells during conditions of BCAA-induced insulin resistance. These findings suggest that increasing catabolic flux of BCAA could have beneficial effects on insulin-resistant skeletal muscle in terms of stimulating glucose uptake. Unexpectedly, PB treatment decreased glycogen content under high BCAA conditions. It is not clear why such differences may be observed compared with previous studies (13) but could reflect differences in experimental conditions. Increased BCAA catabolism has previously been

shown to stimulate fatty acid oxidation and cellular respiration in cultured cells (17); therefore, it is possible that increased glucose uptake, along with decreased glycogen synthesis with PB treatment, could reflect a stimulation of cellular glucose metabolism. These changes could also be linked to alterations in metabolism caused by PB, since there are indications that PB may influence regulation of the pyruvate dehydrogenase complex (PDC) (7). It is also possible that some of the observed changes occurred independently of BCAA, since PB has also been shown to influence other biological pathways, including inhibition of histone deacetylases (16), which could influence glucose utilization. In summary, previous findings of enhanced flux through PDC, and the present observations of enhanced molecular regulators of glucose uptake, indicate that increased muscle glucose utilization may have occurred as a result of PB administration, likely driven by multiple factors, which include BCAA catabolism.

Study limitations and future perspectives. Intracellular levels of BCKA were not measured in these experiments, which would have given further insight into downstream impacts of elevated BCAA on muscle cells, since one mechanism by which BCAAs are thought to cause insulin resistance is via inhibitory effects of 3HIB on insulin signaling and fatty acid metabolism (14). While AS160 phosphorylation has been reported to be a key step in GLUT4 translocation to the cell surface (24), which was used in the present study as an indirect estimation of glucose uptake, direct measures of glucose uptake would have strengthened our findings. Measurements of fatty acid metabolites [as regulators of BCAA catabolism (22)] in the present study would have also allowed us to further investigate the potential links between fatty acid metabolism and insulin resistance due to BCAA. Measuring media BCKA levels in the present study, which would have originated from the cells, did however give us some insights into BCAA metabolism with PB treatment and BCAA exposure levels. For example, under conditions of high BCAA, while PB reduced BCAA concentrations, elevations in BCKA were unaltered, which could indicate some impairments in the BCAA catabolic pathways under conditions of elevated BCAA. Of note was the observation that sustained increases in media BCAA have little influence on basal glutamine and glutamate concentrations, while alanine levels were significantly lower compared with 1× BCAA. While our model in the present study aimed to focus on muscle-specific effects of BCAA on insulin resistance, undoubtedly more detailed analysis, including investigation of the role of muscle-liver and/or muscle-adipose cross talk in the relationship between BCAA and insulin resistance, will ultimately be required to fully understand the full impact of sustained increases in BCAA on amino acid/substrate metabolism.

In conclusion, in the present study we demonstrated that continued exposure of high BCAA to skeletal muscle cells in vitro resulted in impaired intracellular insulin signaling and insulin-stimulated glycogen synthesis, and while PB treatment accelerated BCAA catabolism and activated proteins important for mediating cellular glucose uptake, BCAA catabolism caused further reductions in insulin-stimulated glycogen synthesis. These observations could argue against the use of targeting BCAA catabolism for lowering BCAA concentrations in conditions of insulin resistance in vivo. The findings presented contribute to further insight into regulation of glucose metabolism by BCAA in muscle, and future work using preclinical and human subjects (both healthy volunteers and patient populations) will be required to further increase our understanding of the therapeutic potential of targeting BCAA catabolism in conditions of metabolic dysfunction such as T2DM.

GRANTS

This work was supported by the Medical Research Council Grant MR/P021220/1 as part of the Medical Research Council-Versus Arthritis Centre for Musculoskeletal Aging Research awarded to the Universities of Nottingham and Birmingham.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.S., I.I., B.E.P., P.J.A., and D.J.W. conceived and designed research; H.C. performed experiments; H.C. analyzed data; H.C., K.S., I.I., P.J.A., and D.J.W. interpreted results of experiments; H.C. prepared figures; H.C. drafted manuscript; H.C., K.S., I.I., B.E.P., P.J.A., and D.J.W. edited and revised manuscript; H.C., K.S., I.I., B.E.P., P.J.A., and D.J.W. approved final version of manuscript.

REFERENCES

1. **Asghari G**, **Farhadnejad H**, **Teymoori F**, **Mirmiran P**, **Tohidi M**, **Azizi F.** High dietary intake of branched-chain amino acids is associated with an increased risk of insulin resistance in adults. *J Diabetes* 10: 357–364, 2018. doi:10.1111/1753-0407.12639.
2. **van den Berg EH**, **Flores-Guerrero JL**, **Gruppen EG**, **de Borst MH**, **Wolak-Dinsmore J**, **Connelly MA**, **Bakker SJ**, **Dullaart RP.** Non-alcoholic fatty liver disease and risk of incident type 2 diabetes: Role of circulating branched-chain amino acids. *Nutrients* 11: 705, 2019. doi:10.3390/nu11030705.
3. **Brown DM**, **Jones S**, **Daniel ZC**, **Brearley MC**, **Lewis JE**, **Ebling FJ**, **Parr T**, **Brameld JM.** Effect of sodium 4-phenylbutyrate on Clenbuterol-mediated muscle growth. *PLoS One* 13: e0201481, 2018. doi:10.1371/journal.pone.0201481.
4. **Brunetti-Pierri N**, **Lanpher B**, **Erez A**, **Ananieva EA**, **Islam M**, **Marini JC**, **Sun Q**, **Yu C**, **Hegde M**, **Li J**, **Wynn RM**, **Chuang DT**, **Hutson S**, **Lee B.** Phenylbutyrate therapy for maple syrup urine disease. *Hum Mol Genet* 20: 631–640, 2011. doi:10.1093/hmg/ddq507.
5. **Cummings NE**, **Williams EM**, **Kasza I**, **Konon EN**, **Schaid MD**, **Schmidt BA**, **Poudel C**, **Sherman DS**, **Yu D**, **Arriola Apelo SI**, **Cottrell SE**, **Geiger G**, **Barnes ME**, **Wisinski JA**, **Fenske RJ**, **Matkowskyj KA**, **Kimple ME**, **Alexander CM**, **Merrins MJ**, **Lamming DW.** Restoration of metabolic health by decreased consumption of branched-chain amino acids. *J Physiol* 596: 623–645, 2018. doi:10.1113/JP275075.
6. **David J**, **Dardevet D**, **Mosoni L**, **Savary-Auzeloux I**, **Polakof S.** Impaired skeletal muscle branched-chain amino acids catabolism contributes to their increased circulating levels in a non-obese insulin-resistant fructose-fed rat model. *Nutrients* 11: 355, 2019. doi:10.3390/nu11020355.
7. **Ferriero R**, **Brunetti-Pierri N.** Phenylbutyrate increases activity of pyruvate dehydrogenase complex. *Oncotarget* 4: 804–805, 2013. doi:10.18632/oncotarget.1000.
8. **Flores-Guerrero JL**, **Osté MCJ**, **Kieneker LM**, **Gruppen EG**, **Wolak-Dinsmore J**, **Otvos JD**, **Connelly MA**, **Bakker SJL**, **Dullaart RPF.** Plasma branched-chain amino acids and risk of incident type 2 diabetes: results from the PREVEND prospective cohort study. *J Clin Med* 7: 513, 2018. doi:10.3390/jcm7120513.
9. **Giesbertz P**, **Daniel H.** Branched-chain amino acids as biomarkers in diabetes. *Curr Opin Clin Nutr Metab Care* 19: 48–54, 2016. doi:10.1097/MCO.0000000000000235.
10. **Hernández-Alvarez MI**, **Díaz-Ramos A**, **Berdasco M**, **Cobb J**, **Planet E**, **Cooper D**, **Pazderska A**, **Wanic K**, **O'Hanlon D**, **Gomez A**, **de la Ballina LR**, **Esteller M**, **Palacin M**, **O'Gorman DJ**, **Nolan JJ**, **Zorzano A.** Early-onset and classical forms of type 2 diabetes show impaired expression of genes involved in muscle branched-chain amino acids metabolism. *Sci Rep* 7: 13850, 2017. doi:10.1038/s41598-017-14120-6.
11. **Holecōek M.** Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. *Nutr Metab (Lond)* 15: 33, 2018. doi:10.1186/s12986-018-0271-1.

12. **Holecek M**, **Vodenicarovova M**, **Siman P.** Acute effects of phenylbutyrate on glutamine, branched-chain amino acid and protein metabolism in skeletal muscles of rats. *Int J Exp Pathol* 98: 127–133, 2017. doi:10.1111/iep.12231.
13. **Hu H**, **Li L**, **Wang C**, **He H**, **Mao K**, **Ma X**, **Shi R**, **Oh Y**, **Zhang F**, **Lu Y**, **Wu Q**, **Gu N.** 4-Phenylbutyric acid increases GLUT4 gene expression through suppression of HDAC5 but not endoplasmic reticulum stress. *Cell Physiol Biochem* 33: 1899–1910, 2014. doi:10.1159/000362967.
14. **Jang C**, **Oh SF**, **Wada S**, **Rowe GC**, **Liu L**, **Chan MC**, **Rhee J**, **Hoshino A**, **Kim B**, **Ibrahim A**, **Baca LG**, **Kim E**, **Ghosh CC**, **Parikh SM**, **Jiang A**, **Chu Q**, **Forman DE**, **Lecker SH**, **Krishnaiah S**, **Rabinowitz JD**, **Weljie AM**, **Baur JA**, **Kasper DL**, **Arany Z.** A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. *Nat Med* 22: 421–426, 2016. doi:10.1038/nm.4057.
15. **Kawaguchi T**, **Nagao Y**, **Matsuoka H**, **Ide T**, **Sata M.** Branched-chain amino acid-enriched supplementation improves insulin resistance in patients with chronic liver disease. *Int J Mol Med* 22: 105–112, 2008. doi:10.3892/ijmm.22.1.105.
16. **Khan S**, **Komarya SK**, **Jena G.** Phenylbutyrate and β-cell function: contribution of histone deacetylases and ER stress inhibition. *Epigenomics* 9: 711–720, 2017. doi:10.2217/epi-2016-0160.
17. **Lerin C**, **Goldfine AB**, **Boes T**, **Liu M**, **Kasif S**, **Dreyfuss JM**, **De Sousa-Coelho AL**, **Daher G**, **Manoli I**, **Sysol JR**, **Isganaitis E**, **Jessen N**, **Goodyear LJ**, **Beebe K**, **Gall W**, **Venditti CP**, **Patti ME.** Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism. *Mol Metab* 5: 926–936, 2016. doi:10.1016/j.molmet.2016.08.001.
18. **Lynch CJ**, **Adams SH.** Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat Rev Endocrinol* 10: 723–736, 2014. doi:10.1038/nrendo.2014.171.
19. **Mardinoglu A**, **Gogg S**, **Lotta LA**, **Stančáková A**, **Nerstedt A**, **Boren J**, **Blüher M**, **Ferrannini E**, **Langenberg C**, **Wareham NJ**, **Laakso M**, **Smith U.** Elevated plasma levels of 3-hydroxyisobutyric acid are associated with incident type 2 diabetes. *EBioMedicine* 27: 151–155, 2018. doi:10.1016/j.ebiom.2017.12.008.
20. **Miyake T**, **Abe M**, **Furukawa S**, **Tokumoto Y**, **Toshimitsu K**, **Ueda T**, **Yamamoto S**, **Hirooka M**, **Kumagi T**, **Hiasa Y**, **Matsuura B**, **Onji M.** Long-term branched-chain amino acid supplementation improves glucose tolerance in patients with nonalcoholic steatohepatitis-related cirrhosis. *Intern Med* 51: 2151–2155, 2012. doi:10.2169/internalmedicine.51.7578.
21. **O'Connell TM.** The complex role of branched chain amino acids in diabetes and cancer. *Metabolites* 3: 931–945, 2013. doi:10.3390/metabo3040931.
22. **Shimomura Y**, **Murakami T**, **Nakai N**, **Nagasaki M**, **Harris RA.** Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise. *J Nutr* 134, Suppl: 1583S–1587S, 2004. doi:10.1093/jn/134.6.1583S.
23. **Solerte SB**, **Fioravanti M**, **Locatelli E**, **Bonacasa R**, **Zamboni M**, **Basso C**, **Mazzoleni A**, **Mansi V**, **Geroutis N**, **Gazzaruso C.** Improvement of blood glucose control and insulin sensitivity during a long-term (60 weeks) randomized study with amino acid dietary supplements in elderly subjects with type 2 diabetes mellitus. *Am J Cardiol* 101, 11A: 82E–88E, 2008. doi:10.1016/j.amjcard.2008.03.006.
24. **Stöckli J**, **Davey JR**, **Hohnen-Behrens C**, **Xu A**, **James DE**, **Ramm G.** Regulation of glucose transporter 4 translocation by the Rab guanosine triphosphatase-activating protein AS160/TBC1D4: role of phosphorylation and membrane association. *Mol Endocrinol* 22: 2703–2715, 2008. doi:10.1210/me.2008-0111.
25. **White PJ**, **Lapworth AL**, **An J**, **Wang L**, **McGarrah RW**, **Stevens RD**, **Ilkayeva O**, **George T**, **Muehlbauer MJ**, **Bain JR**, **Trimmer JK**, **Brosnan MJ**, **Rolph TP**, **Newgard CB.** Branched-chain amino acid restriction in Zucker-fatty rats improves muscle insulin sensitivity by enhancing efficiency of fatty acid oxidation and acyl-glycine export. *Mol Metab* 5: 538–551, 2016. doi:10.1016/j.molmet.2016.04.006.
26. **Xiao C**, **Giacca A**, **Lewis GF.** Sodium phenylbutyrate, a drug with known capacity to reduce endoplasmic reticulum stress, partially alleviates lipid-induced insulin resistance and β-cell dysfunction in humans. *Diabetes* 60: 918–924, 2011. doi:10.2337/db10-1433.
27. **Yamada C**, **Kondo M**, **Kishimoto N**, **Shibata T**, **Nagai Y**, **Imanishi T**, **Oroguchi T**, **Ishii N**, **Nishizaki Y.** Association between insulin resistance and plasma amino acid profile in non-diabetic Japanese subjects. *J Diabetes Investig* 6: 408–415, 2015. doi:10.1111/jdi.12323.
28. **Yoneshiro T**, **Wang Q**, **Tajima K**, **Matsushita M**, **Maki H**, **Igarashi K**, **Dai Z**, **White PJ**, **McGarrah RW**, **Ilkayeva OR**, **Deleye Y**, **Oguri Y**, **Kuroda M**, **Ikeda K**, **Li H**, **Ueno A**, **Ohishi M**, **Ishikawa T**, **Kim K**, **Chen Y**, **Sponton CH**, **Pradhan RN**, **Majd H**, **Greiner VJ**, **Yoneshiro M**, **Brown Z**, **Chondronikola M**, **Takahashi H**, **Goto T**, **Kawada T**, **Sidossis L**, **Szoka FC**, **McManus MT**, **Saito M**, **Soga T**, **Kajimura S.** BCAA catabolism in brown fat controls energy homeostasis through SLC25A44. *Nature* 572: 614–619, 2019. doi:10.1038/s41586-019-1503-x.
29. **Yoon MS.** The emerging role of branched-chain amino acids in insulin resistance and metabolism. *Nutrients* 8: 405, 2016. doi:10.3390/nu8070405.
30. **Yoon MS**, **Choi CS.** The role of amino acid-induced mammalian target of rapamycin complex 1(mTORC1) signaling in insulin resistance. *Exp Mol Med* 48: e201, 2016. doi:10.1038/emm.2015.93.
31. **Zhou M**, **Shao J**, **Wu CY**, **Shu L**, **Dong W**, **Liu Y**, **Chen M**, **Wynn RM**, **Wang J**, **Wang J**, **Gui WJ**, **Qi X**, **Lusis AJ**, **Li Z**, **Wang W**, **Ning G**, **Yang X**, **Chuang DT**, **Wang Y**, **Sun H.** Targeting BCAA catabolism to treat obesity-associated insulin resistance. *Diabetes* 68: 1730–1746, 2019. doi:10.2337/db18-0927.