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ORIGINAL ARTICLE

Chronic activation of mTOR complex 1 by branched chain amino acids and organ hypertrophy

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Abstract The mitochondrial branched chain aminotransferase-deficient mouse model (BCATm KO), which exhibits elevated plasma and tissue branched chain amino acids (BCAAs), was used to study the effect of BCAAs on mammalian target of rapamycin complex 1 (mTORC1) regulation of organ size. BCATm is the first enzyme in the BCAA catabolic pathway. BCATm KO mouse exhibited hypertrophy of heart, kidneys, and spleen. On the other hand, the mass of the gastrocnemius was reduced relative to body mass. Feeding the mice with a diet supplemented with rapamycin prevented the enlargement of the heart and spleen, suggesting that mTORC1 is the mediator of these effects. Consistently, enlargement of these organs was accompanied by the activation of mTORC1 complex as evidenced by enhanced levels of S6 and 4E-BP1 phosphorylation. HSP20, HSP27 and GAPDH were also increased in the heart but not gastrocnemius, consistent with mTORC1 activation. Liver, however, displayed no weight difference between the KO and the wild-type mice despite the highest activation level of mTORC1 complex. These observations suggest that the anabolic effect of mTORC1 activation at the organ level by BCAAs and inhibition by rapamycin are complex phenomenon and tissue-specific. In addition, it suggests that rapamycin can be used to counter hypertrophy of the organs when activation of mTORC1 is the underlying cause.

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Introduction

The branched chain amino acids (BCAAs), valine, isoleucine and leucine in particular, act as nutrient signals that promote protein synthesis in skeletal muscle, heart, adipose tissue, liver (Kimball and Jefferson 2006; Anthony et al. 2001, 2000) and insulin release (Malaisse et al. 1982; Li et al. 2003). Metabolically they are thought to be important donors of nitrogen for Ala and Gln synthesis in skeletal muscle (Hutson et al. 1978; Garber et al. 1976) and for Glu neurotransmitter synthesis in the central nervous system (Bixel et al. 1997; Hutson et al. 2005; Yudkoff et al. 2005). The anabolic effects of BCAAs and their quantitative importance (40 % of indispensable amino acid requirement) have led to their use in treating medical conditions and as nutritional supplements. For example, Leu supplementation is shown to prevent hepatocarcinogenesis, prolong survival of patients with cirrhosis (Kawaguchi et al. 2013), be beneficial for glycemic control in chronic hepatitis C patients with insulin resistance (Takeshita et al. 2012), and reduce exercise-induced muscle damage (Howatson et al. 2012). Furthermore, BCAA supplements are widely used by body builders (Thalacker-Mercer et al. 2009). Therefore, a detailed study of the effects of these amino acids on different organs including muscles is warranted.

The branched chain amino acid catabolism involves two initial common enzymatic steps; therefore, the dietary intake of one BCAA impacts the catabolism of all three. The first catabolic step is reversible transamination catalyzed by the branched chain aminotransferase (BCAT) isozymes (mitochondrial BCATm and cytosolic BCATc).

The BCATs are vitamin B6 or pyridoxal phosphate (PLP)-dependent enzymes (Hutson et al. 1992, 1995) that are responsible for reversible transfer of the α-amino group from a BCAA to α-ketoglutarate (α-KG) to form Glu and the respective branched chain α-keto acids (BCKAs), α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), and α-ketoisovalerate (KIV) (Ichihara and Koyama 1966; Davoodi et al. 1998). BCATm is expressed ubiquitously except in the liver of rodents, while BCATc expression is limited to the brain, peripheral nerves and immune cells (Hutson et al. 2005). Dysregulation of the BCAA catabolic pathway at the second step leads to excess levels of BCAAs and their BCKAs resulting in Maple Syrup Urine Disease (Muelly et al. 2013) which, if untreated, results in severe neural dysfunction and death. The disruption of the catabolic pathway at the first step (BCATm KO mouse), which leads to elevated body BCAAs including Leu without elevated BCKAs, does not produce the MSUD phenotype (She et al. 2007) and is not known to be associated with any disease. Nonetheless, it would be important to determine if increased BCAA levels can produce any specific pathology given their widespread use in various conditions and as exercise and body building supplements.

There is a considerable body of information describing the regulation of mammalian target of rapamycin complex 1 (mTORC1) by Leu for a recent review see Jewell et al. (2013). These studies rely on cell culture models where it is easier to manipulate cell signaling machinery. Although, organ enlargement is shown to be associated with mTORC1 activation, a detailed study comparing responsiveness of different organs to mTORC1 activation is lacking. Deletion of BCATm gene, however, removes the first step in catabolism of BCAAs leading to a chronic increase in the concentrations of these amino acids and activation of mTORC1 (She et al. 2007). Despite high BCAA concentrations, 6-h fasted BCATm KO mice had lower plasma leptin (ninefold reduction), insulin (threefold reduction), and norepinephrine (twofold reduction, fed state), while IGF-1 and thyroxine of the fed BCATm KO mice remained the same as the wild-type mice. In spite of lower insulin concentration, the concentration of glucose was not altered by BCATm deletion in the fed mice. In addition, BCATm KO mice resist a high-fat diet, as they do not become obese, and are more insulin sensitive. The BCATm KO mouse provides an excellent model to study the effect of chronic activation of mTORC1 by leucine in different tissues with regard to the extent of activation and the relationship between this activation and relative organ size under in vivo conditions. In addition, this mouse model provides a means to determine if BCAA effects originate from BCAAs or their metabolites. The original paper describing the BCATm mouse model showed the chronic effect of elevated leucine on the relative organ size in male mice on a high-fat diet (She et al. 2006). Kidneys showed increased mass while epididymal fat was reduced. All other organs were similar in size. In this study, we investigated whether or not organ size in the female mice follows the same pattern as the male mice and its relation to mTORC1 activation. Our findings show that mTORC1 activation was sufficient to produce larger hearts and spleens, while insufficient to cause liver hypertrophy. In addition, up regulation of small heat shock proteins of HSP27 and HSP20 was observed in the heart but not in the gastrocnemius of BCATm KO mice which is in agreement with published results showing protective effects of HSPs in heart hypertrophy (Toko et al. 2008). Also, up regulation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was seen which is consistent with mTORC1 activation (Cairns et al. 2011).

Materials and methods

Animals and diet treatments

All animal experiments were approved by the IACUC at Virginia Tech. The singly caged female mice (C57 Black/6) were kept on a choice amino acid diet of Low BCAA (LBCAA), and Normal BCAA (NBCAA), (access to both LBCAA and NBCAA diets), until 6 weeks of age. The NBCAA diets consisted of 12, 8, and 8 percent Leu, Ile, and Val, respectively, while LBCAA contained 0.3, 0.2, and 0.2 percent of these amino acids (Table 1). All diets were purchased from Research Diets Inc. The control NBCAA diet for the rapamycin experiments contained sham capsules only. The preparation of rapamycin capsules was performed by Quality Lab Products Inc. as described previously (Harrison et al. 2009). The experimental diet contained 14 mg rapamycin per 1 kg of the diet. Based on the paper describing the microencapsulation of rapamycin, the plasma concentration of rapamycin is about 60 ng/ml. Prior to the experiments, the mice with n of 5–10 were fed LBCAA diet for 3 days then the diet was switched to NBCAA with or without rapamycin for 10 days. The mice were killed by cervical dislocation following a 6-h fast on day 10. The collected organs were weighed, freeze-clamped and stored at −80 °C for further analysis. Prior to freeze-clamping, the heart samples were cut into smaller pieces and immersed in PBS to remove blood.

Indirect calorimetry

VCO_2, VO_2, and movement were recorded using TSE Lambaster Calorimetry system which was calibrated using a defined mixture of O_2 and CO_2. The mice were placed in the TSE from day 6 to day 9 of the 10-day NBCAA ± rapamycin diet treatment. Measurements were

Table 1 Diet composition for normal branched chain amino acid diet (NBCAA) and low branched chain amino acid diet (LBCAA) used throughout this study

	NBCAA		LBCAA	
	g %	kcal %	g %	kcal %
Protein	16	16.6	15.4	16
Carbohydrate	69.3	71.7	69.8	72.2
Fat	5.1	11.8	5.1	11.9
Total		100		100
kcal/gm	3.87		3.87	
Ingredient	gm	kcal	gm	kcal
L-Arginine	8.27		9.57	
L-Histidine–HCl–H_2O	6		6.95	
L-Isoleucine	8		0.2	
L-Leucine	12		0.3	
L-Lysine–HCl	14		16.21	
DL-Methionine	6		6.95	
L-Phenylalanine	8		9.26	
L-Threonine	8		9.26	
L-Tryptophan	2		2.32	
L-Valine	8		0.2	
L-Alanine	10		11.58	
L-Asparagine–H_2O	10		11.58	
L-Cystine	4		4.63	
L-Glutamic acid	10		11.58	
L-Glutamine	10		11.58	
Glycine	10		11.58	
L-Proline	10		11.58	
L-Serine	10		11.58	
L-Tyrosine	4		4.63	
Total L-amino acids	158.27	633	151.54	606
Corn starch	300	1200	300	1200
Maltodextrin 10	125	500	125	500
Sucrose	250	1000	250	1000
Cellulose	50	0	50	0
Soybean oil	50	450	50	450
Mineral mix S 10001	35	0	35	0
Sodium bicarbonate	7.5	0	7.5	0
Vitamin Mix V10001	10	40	10	40
Choline bitartrate	2	0	2	0
Diammonium citrate	0	0	0	0
Total	987.9791	3823	981.09	3796
Na (gm)	4.5		4.5	
Nitrogen(gm)	22.1		22.1	

The NBCAA diet without rapamycin was used as the control diet and with 14 mg rapamycin per 1 kg was used as the experimental diet

taken every 15 min. The chambers were equipped with infrared beams to measure total, areal and vertical movements. Total activity corresponded to fine movements (interception of a single beam) plus the areal movement (interception of more than one beam). Vertical movement corresponded to beam break during standing and reaching food, hanging from the bars, etc.). Respiratory exchange ratio, RER, was calculated as the ratio of VCO_2 over VO_2, and energy expenditure, EE, (kJ/h) was calculated using the equation $4.1868 \times [3.815 + (1.232 \times \text{RER})] \times VO_2$, with VO_2 defined as milliliters of O_2 per h (Longo et al. 2010). Energy expenditure was reported per gram of fat-free mass per hour. Body composition was determined using a Bruker LF 90 NMR before (day 6 on the NBCAA diet) and after placing the mice in the chambers (day 9 on NBCAA diet).

Tissue extraction and western blotting

Frozen tissues were immersed in liquid nitrogen and ground with a mortar and pestle. The tissue powders were suspended in protein extraction buffer (PEB) which consisted of 25 mM HEPES pH 7.4, 0.4 % CHAPS, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 5 mM benzamidine, Cal Biochem proteinase Inhibitor cocktail III (as per recommendation of the manufacturer), 50 mM thiamine pyrophosphate, 50 mM β-glycerophosphate, 94 nM microcystin, 0.71 mM sodium ortho-vanadate, 100 mM potassium fluoride, and 1 mM 2-chloroisocaproate. The tissue lysates were then extracted with a bullet blender and centrifuged at 12,000× g for 20 min at 4 °C. The bullet blender was set at 8 for all samples. Blending time for the heart and gastrocnemius was 8 min while four min were used for the spleen and liver. The supernatants were removed and the protein concentrations were determined using a BCA protein assay kit from Thermo Scientific. To verify the accuracy of the protein measurements, an equal amount of each sample was loaded onto SDS-PAGE and stained with Coomassie R-250. To compare the effect of high leucine concentrations on mTORC1 signaling in different tissues, total protein along with phosphoprotein levels for S6 ribosomal protein (Ser 235/236), mTOR (Ser 2448), and 4E-BP1 (Thr37/46) were assessed by Western blotting using rabbit monoclonal antibodies. Relative levels of total and phosphorylated forms of AMPK (Thr 172) and actin were also determined by Western blot. All antibodies were purchased from Cell Signaling except for affinity purified BCATm antibody (Suryawan et al. 1998; Wallin et al. 1990), horse radish peroxidase conjugated donkey anti-rabbit (Jackson Immuno Research Labs, West Grove, PA) secondary antibody, and caspase-3. Recombinant caspase-3 was produced and purified according to the procedure described in reference (Abhari and Davoodi 2008). Phosphate buffered saline dialyzed caspase-3 was used to immunize white New Zealand Rabbits according to the procedure described for Ribi Adjuvant System (RAS, Sigma-Aldrich). The transfer to PVDF membrane was performed at a constant current

of 300 mA for 1-h using Towbin buffer. The membranes were blocked in a 2 % BSA solution of Tris-buffered Saline (TBS) containing 0.1 % Tween-20. Antibodies were added to the blocking solution according to the manufacturer's protocol, and the membranes were exposed to the antibodies overnight at 4 °C. ImageJ software was used to quantify the band intensities. The average band intensity of the wild-type samples was assigned as 100 %, and the relative intensity of all bands was compared to the mean. Following normalization, the intensity of each band resulting from the phosphoprotein was divided to the intensity of the band corresponding to total protein to obtain the ratio. To verify equal loading, all of the blots were stained with 0.1 % R-250 Coomassie blue dye in 40 % methanol and 10 % acetic acid. The membranes were destained with the same solution without the dye followed by a solution of 90 % methanol and 5 % acetic acid. Given the down regulating effect of rapamycin treatment on actin protein in heart, Coomassie stained blot was included to verify loading. In the case of gastrocnemius, actin antibody did not recognize this protein which prompted us to use Coomassie stained blot as a loading control.

Plasma amino acid concentration

Plasma amino acid concentrations were determined as described previously (Wu and Knabe 1994). Briefly, high-pressure liquid chromatography was used and the amino acids were derivatized to their *O*-phthaldialdehyde forms and eluted by sodium acetate methanol gradient on a Supelcosil™ LC-18 column (15 cm × 4.6 mm, 3 μm, Sigma, St. Louis, MO). Perchloric acid (1.5 M) was used to deproteinize the samples then neutralized with a 2 M solution of potassium carbonate.

Two-dimensional gel electrophoresis

Heart samples were ground using a mortar and pestle while keeping the samples cold using liquid nitrogen. The proteins were extracted from the ground powder using Ready-Prep Protein Extraction Kit (Soluble/Insoluble) from Bio-Rad according to the manufacturer's directions. The protein concentration was determined by Amido Black assay using BSA as the protein standard (Conway and Hutson 2000). IPGPHOR from GE Life Sciences was used to perform the first dimension in 7 cm nonlinear 3–10 IPG strips from Biorad. The following steps were followed to perform the first dimension: Step 1, 250 volts-hour at 500 V; Step 2, 500 volts-hour at 1000 V, 2000 volts-hour (gradient) at 8000 V, 23000 volts-hour at 8000 V. The second dimension was performed at constant 120 volts for 70 min. The gels were stained with G-250 and the picked spots were identified by AB SCIEX 4800 TOF/TOF Tandem Mass spectrometer using the Virginia Tech Mass Spectrometry Incubator (Department of Biochemistry, Virginia Tech).

Statistical analysis

Two-tailed non-paired *t* test was used to determine the significant differences between the BCATm−/− and wild-type mice. Values are means of ±SE with p values <0.05 being significant.

Results

Animals had access to free amino acid diets of both LBCAA (0.3, 0.2, and 0.2 % of Leu, Ile, and Val) and NBCAA (12, 8, and 8 % of Leu, Ile, and Val) until 3 days prior to the start of the experiment. The female BCATm KO mice preferred LBAA diet over NBCAA diet, whereas the WT mice preferred NBCAA over LBCAA, so they were very similar to their male counterparts (She et al. 2007). Nonetheless, considerable mouse to mouse variation existed in their daily diet choice affecting plasma BCAA concentrations. Therefore, we fed female BCATm KO mice LBAA diet for 3 days prior to administering the test diet. At the start of the experiment, both the WT and BCATm KO mice were fed the NBCAA diet with or without rapamycin. Microencapsulation of rapamycin protected the drug in the gastrointestinal tract allowing its delivery through the diet (Harrison et al. 2009). The added benefit of dietary supplementation was to maintain a continuous presence of the drug in the body and increase drug dose in plasma during feeding with minimal stress to the animals. Given the activation of mTORC1 by BCAAs, and inhibition of mTORC1 by rapamycin, this drug was used to determine BCAA-mediated mTORC1 effects.

Plasma amino acid concentrations of the female BCATm KO mice

The plasma BCAA concentrations of the female BCATm KO mice fasted for 6 h were higher in mice fed the NBCAA diet than in wild-type mice (Table 2). Unlike BCATm, which is ubiquitously expressed, BCATc expression is limited to the brain and immune cells (Hutson et al. 2005; Castellano et al. 2007) and thus cannot fully compensate for BCATm loss causing such a drastic increase in BCAA concentration. In addition to BCAAs, other large neutral amino acids, Trp, Met, and Thr, were also increased in plasma, but Tyr was not elevated. Inclusion of rapamycin in the diet did not normalize the concentration of any of the amino acids in the plasma of the KO mice despite the fact that it significantly reduced BCAA concentration in the blood though not to WT concentrations. The original

Table 2 Plasma amino acid concentrations for the female wild type and BCATm KO mice on NBCAA diet without or with rapamycin

Plasma amino acids	Wild type	BCATm KO	Wild type + rapamycin	BCATm KO + rapamycin
LEU	50 ± 6	2562 ± 119*	55 ± 4	1541 ± 92*#
ILE	33 ± 4	2587 ± 198*	38 ± 4	1643 ± 115*#
VAL	72 ± 9	3965 ± 244	84 ± 9	2542 ± 187*#
MET	18 ± 1	29 ± 2*	18 ± 2	26 ± 2*
TRP	26 ± 4	79 ± 7	26 ± 4	54 ± 8
TYR	39 ± 3	36 ± 4	41 ± 4	31 ± 3
ALA	299 ± 35	210 ± 15*	297 ± 24	231 ± 16*
ARG/TAU	342 ± 51	450 ± 50	314 ± 28	458 ± 56
CIT	54 ± 18	73 ± 7	45 ± 4	60 ± 4
THR	122 ± 12	216 ± 18*	145 ± 15	191 ± 13*
GLY	171 ± 34	245 ± 30	163 ± 29	207 ± 30
GLN	499 ± 88	421 ± 40	565 ± 79	428 ± 40
SER	69 ± 6	92 ± 9	77 ± 10	80 ± 9
ASN	52 ± 5	43 ± 4	47 ± 4	48 ± 3
GLU	80 ± 11	45 ± 4*	80 ± 8	63 ± 8
ASP	45 ± 6	25 ± 4*	37 ± 3	33 ± 8
LYS	92 ± 13	122 ± 14	97 ± 14	100 ± 19

Statistically significant differences ($p < 0.05$, $n = 5$–7), between WT and KO on NBCAA diet and WTs on NBCAA and KOs on NBCAA with rapamycin are marked with * KOs on NBCAA with and without rapamycin are shown by #

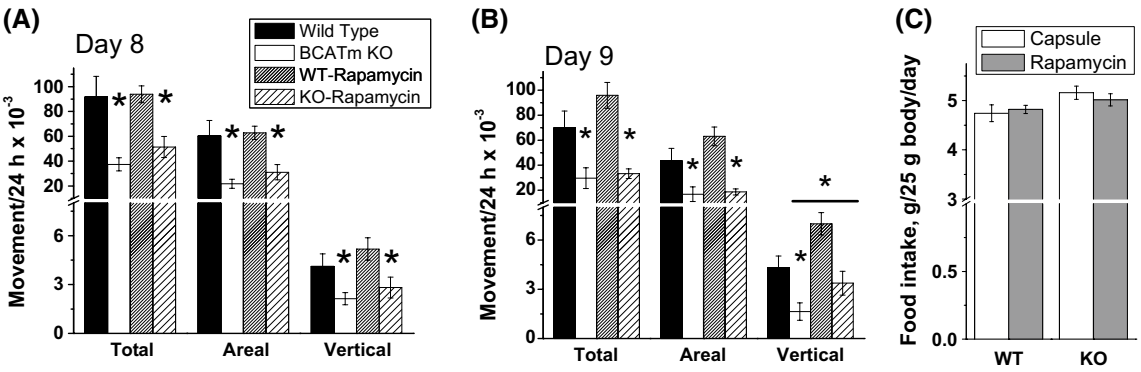

Fig. 1 Movement and food intake for the wild type (WT) and BCATm KO mice provided either normal branched chain amino acid (NBCAA) diet or NBCAA diet with microencapsulated rapamycin. **a** Twenty-four hour movement of the mice was recorded automatically using TSE Lambaster Calorimetry system. Areal movement included beam breaks by the mice movement on *X* and *Y* directions, while the vertical movement included beam break on *Z* direction such as reaching for the food compartment and hanging from the bars. **b** Food intake reported in grams per 25 g of body mass averaged over 9 days. The number of mice per group was 5–10 and *p* value ≤0.05 was considered statistically significant

paper describing the BCATm KO mice provided convincing evidence that a futile cycle of protein synthesis and degradation occurs (She et al. 2007). Chronic activation of mTORC1, caused by elevated BCAA, increases protein synthesis accompanied by a higher demand for amino acids which is partly provided by enhanced protein degradation. Reduction of mTORC1 activity by rapamycin would decrease the demand for amino acids and thus protein degradation. Plasma reduction of BCAAs in the rapamycin-treated BCATm KO mice was observed for other amino acids like Trp, Thr, Gly, Met and others, some statistically significant, some not, and is consistent with this explanation. (Hutson et al. 2005; Castellano et al. 2007).

Improved movement and energy expenditure of rapamycin-treated BCATm KO mice

Male BCATm KO mice were resistant to high-fat diet induced obesity due to enhanced energy expenditure despite the fact that their areal and vertical activity remained the same as wild-type mice (She et al. 2007). To determine if the female mice also exhibited elevated energy expenditure without a change in activity, we used the TSE system to determine energy expenditure and activity of the female BCATm KO and WT animals on the normal BCAA diet (NBCAA) with or without encapsulated rapamycin. Figure 1A shows approximately 90,000 total movements

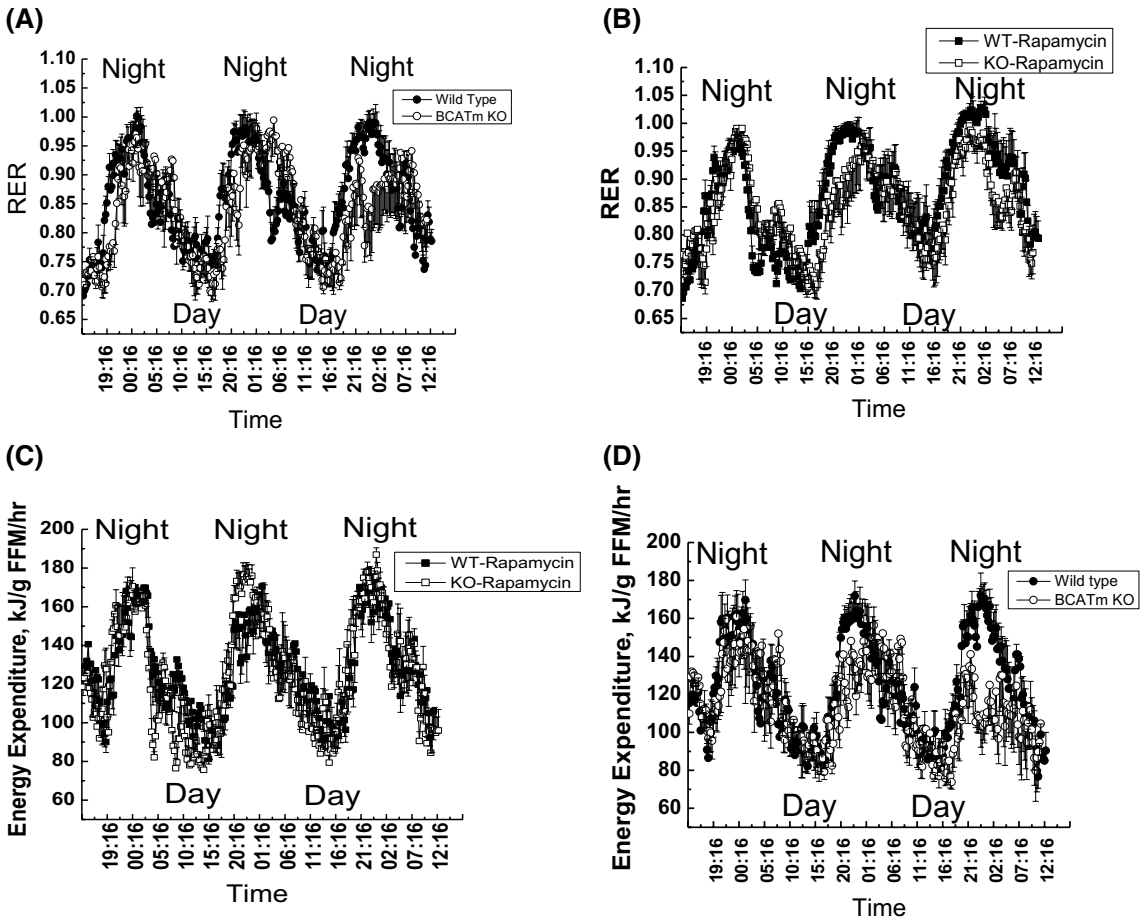

Fig. 2 Indirect calorimetry data for the wild type (WT) and BCATm KO mice on either NBCAA diet or NBCAA diet containing microencapsulated rapamycin were obtained using a TSE Lambaster Calorimetry system. Respiratory exchange ratio (RER) for the wild type and BCATm KO mice on NBCAA diet (**a**), or on NBCAA diet with microencapsulated rapamycin (**b**) are plotted for days of 7 through 9. Energy expenditure in kilojoule per grams of fat-free mass for the wild type and BCATm KO mice on NBCAA diet (**c**), or on NBCAA diet with microencapsulated rapamycin (**d**), are plotted for days 7 through 9. Results were calculated using the equation provided in "Materials and methods". Measurements were taken from five mice per group every 15 min for approximately 3 days. A p value ≤ 0.05 was considered statistically significant

per 24 h for the wild type and 40,000 for the KO mice indicating a more than 50 % reduction in total activity on the 8th day of the experiment. This reduction in activity occurred in all types of movement, areal, fine (not shown) and vertical (rising to upright position). A similar trend was observed on the 9th day of the treatment. Because the food compartment was at the top of the cage, animals had to stand to feed. Considering that food intake was equal or slightly higher for the KO mice compared to wild type (Fig. 1c), the lower number of vertical movements of the KO mice is likely due to reduced acrobatic movement like hanging from the horizontal bars at the top of the cage or vertically oriented cyclic movements.

Energy expenditure and RER were also compared for the wild type and BCATm KO mice on NBCAA diet with and without rapamycin. The KO mice exhibited similar RER and energy expenditure (Fig. 2a, c) on days 7 and 8 of the NBCAA diet treatment as the wild-type mice. During this period RER peaked at about 1.0 at night and dropped to 0.7 during the day. The average night time RER for WT mice on 9th day of treatment was 0.93 ± 0.01 ($n = 5$, $p < 0.01$) while for the KO was 0.87 ± 0.01 ($n = 5$, $p < 0.01$) showing a significant difference from the wild-type mouse values. Inclusion of rapamycin in the diet partly prevented the RER reduction for the BCATm KO. The lower RER for the KO mice suggests small differences in carbohydrate oxidation as a fuel source. A similar trend was observed for the energy expenditure. The average night time energy expenditures on the 7th day of treatment were 134 ± 3 and 129 ± 2 kJ/g FFM/h ($n = 5$, $p < 0.01$) for the wild type and KO mice on the rapamycin-free diet. This value increased slightly to 140 ± 3 for the WT mice by the 9th day of the treatment, while it was decreased to 111 ± 2 kJ/g FFM/h for the BCATm KO mice. On the rapamycin diet, however,

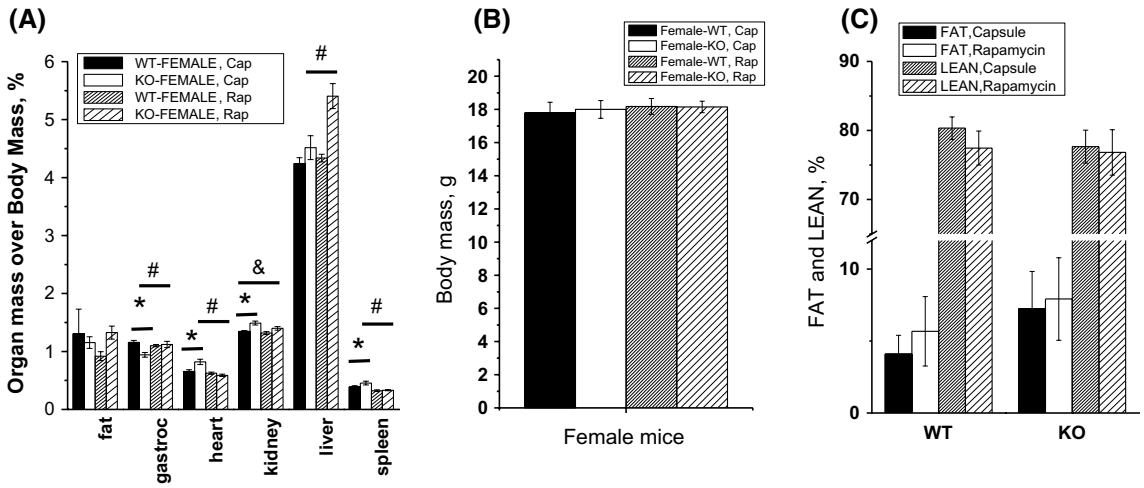

Fig. 3 Relative organ mass, body mass, and composition of wild type (WT) and BCATm KO mice on either NBCAA diet or NBCAA diet with microencapsulated rapamycin. Statistically significant differences, $p \leq 0.05$ ($n = 6$–10) between WT and KO on NBCAA diet, KOs on NCAA with and without rapamycin, and KO on NBCAA with rapamycin and WT on NBCAA are shown by *asterisk*, *hash*, and *ampersand*, respectively

the average night time energy expenditure registered similar numbers of 146 ± 3 and 142 ± 3 kJ/g FFM/h for the WT and BCATm KO mice on day 9. Prevention of energy expenditure reduction for the BCATm KO mice by rapamycin coincided with a slight increase in vertical movement (Fig. 2b).

Organ mass in BCATm KO and WT mice with and without dietary rapamycin

Previously published (Hutson and Harper 1981) and unpublished studies (Hutson et al.) have shown that plasma concentrations of BCAA are reflected in all tissues except brain where the blood brain barrier limits uptake, yet the concentrations are still elevated 2–3 times over the control values. In order to compare the effect of high BCAA levels on organ mass, 6-week-old female KO mice were fed the LBCAA diet for 3 days to equalize plasma BCAA concentrations. Then, they were fed the NBCAA diet to maintain constant high concentrations of BCAA in the plasma. Following 10 days on the NBCAA diet (±rapamycin), organ masses of the mice were measured and normalized to body mass (Fig. 3a). Compared to WT tissues, KO heart with a 25 % showed the highest weight increase; while spleen and kidneys with 18 and 10 % increases, respectively, appeared less responsive than heart. Despite elevated BCAA concentrations, gastrocnemius muscles in the KO mice were smaller than in the wild-type mice. It is surprising that the gastrocnemius muscle was smaller given the known effects of BCAAs on skeletal muscle protein synthesis and the lack of difference in male animals (She et al. 2007). Inclusion of rapamycin normalized the tissue weight for the heart, gastrocnemius, and spleen while it did not have any effect on the kidneys. Lack of a rapamycin effect on the kidneys suggests that hypertrophy of this organ is not mediated by mTORC1 and perhaps is the result of increased work caused by high plasma BCAA concentrations. Despite the changes in relative mass of the organs, body mass and composition for the KO mice remained similar to their WT counterparts (Fig. 3b, c), so in this respect female KOs are different from the KO male mice (She et al. 2007).

Tissue-dependent activation of mTORC1 by BCAAs

It has been shown by a number of laboratories that elevated leucine activates mTORC1 complex (Kimball et al. 2000; Reiter et al. 2004; SR et al. 1999; Gulati et al. 2008), reflected by increased phosphorylation of 4E-BP1 and S6 kinase and its substrate S6 protein. The ultimate outcome of mTORC1 activation is an enhanced rate of protein translation and synthesis. Gastrocnemius muscles of the male BCATm KO mice showed enhanced mTORC1 activation upon refeeding (She et al. 2007). However, gastrocnemius muscle of the 12-h fasted male BCATm KO mice had the same level of 4E-BP1 and S6K phosphorylation as the wild-type mice (She et al. 2007). Twelve hour fasting is long for a mouse. Thus, we decided to reduce the fasting time to 6 h. In addition, we sought to investigate the activation status of the female BCATm KO mice not only in the gastrocnemius, but also other tissues and determine if there was a correlation between organ size and BCAA-mediated mTORC1 activation.

The phosphorylation state of mTOR, S6, and 4E-BP1 proteins in heart, gastrocnemius muscle, liver, and spleen

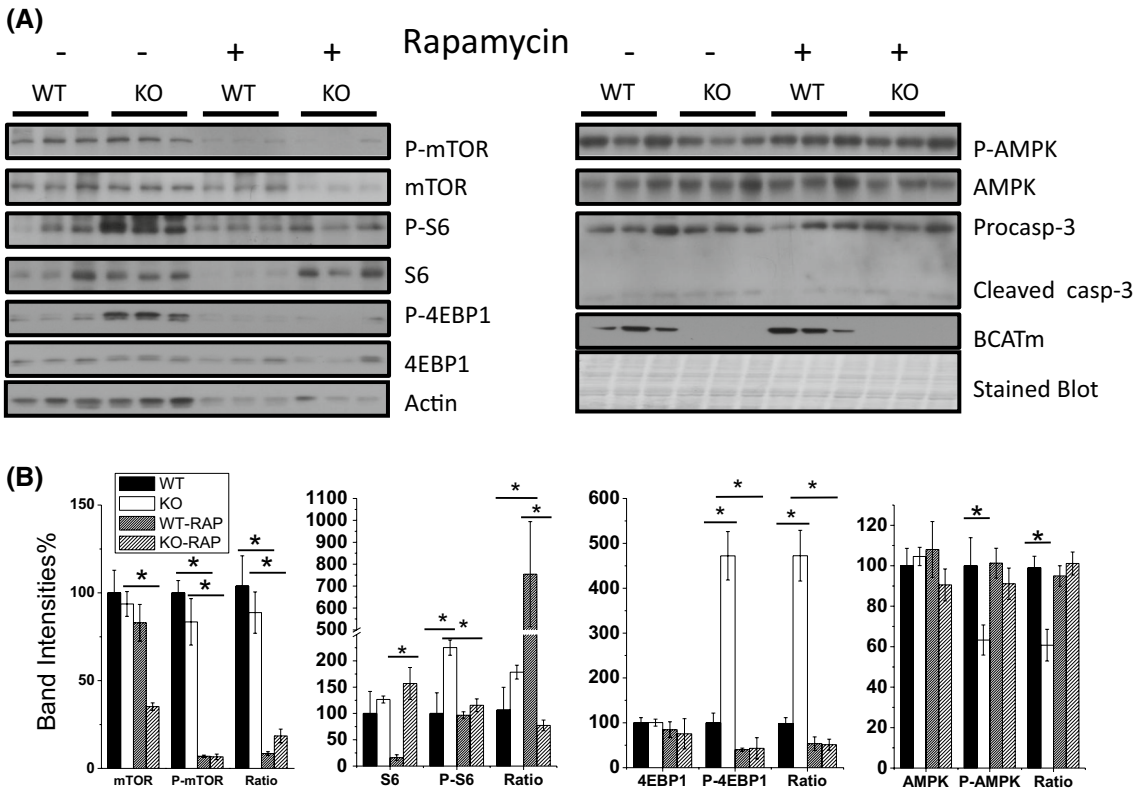

Fig. 4 Activation of mTORC1 signaling pathway in heart muscle of the BCATm KO mice determined by Western blotting (**a**), and quantification of the protein bands from the three samples in the Western blots by ImageJ (**b**). Proteins were: mTOR, S6, 4E-BP1, and AMPK, **b**. Minimal cleavage of procaspase-3 (Procasp-3) was observed in all samples, suggesting the presence of basal apoptosis in this tissue. Given down regulation of actin upon rapamycin treatment in heart, the PVDF membrane was stained with Coomassie blue to ensure equal loading. A p value ≤ 0.05 was considered statistically significant

of the wild type and BCATm KO female mice on NBCAA diet containing either sham capsule or microencapsulated rapamycin was investigated following a short fast (6 h). Western blots for heart are shown in Fig. 4a and quantified in Fig. 4b. Phosphorylation of S6 and 4E-BP1, which represent the active (S6) and inactive (4E-BP1) forms of each protein, was increased in BCATm KO compared to WT mice and normalized by rapamycin. Given the reduction of the total S6 protein in the WT mice upon rapamycin treatment, the ratio of the phosphorylated from over total form increased dramatically, which appears confusing at the first glance. However, the active form of the protein is the phosphorylated form which represents the level of mTORC1 activation and the result is consistent with down regulation of mTORC1 activity in both the wild type and BCATm KOs treated with rapamycin. Interestingly, treatment of the KO mice did not reduce P-S6 below the wild-type level. Perhaps a healthy minimal phosphorylation of S6 is needed for the normal heart function. Unlike S6, the total 4E-BP1 protein remained constant from one group to another resulting in a similar pattern for the ratio of the phosphorylated over total form as the phosphorylated 4E-BP1 protein in WT and KO mice. Given that the active form of 4E-BP1 is the unphosphorylated form, reduced P-4E-BP1 translates into enhanced inhibition of protein translation consistent with the normalization of the heart size of the rapamycin-treated BCATm KO mice. Despite normalization of S6 and 4E-BP1 phosphorylation in rapamycin-treated BCATm KO mice, mTOR phosphorylation was reduced below the values observed for the control wild-type mice. Compared to the WT heart tissue, rapamycin treatment reduced total S6 protein in the WT and mTOR protein in the KOs without affecting 4E-BP1. The study, which we based our rapamycin treatment upon, only measured S6 protein levels in adipose tissue and did not observe any change (Harrison 2009). We could not find any other study similar to ours to compare the effect of rapamycin on total mTORC1 and S6 protein levels. Nonetheless, treatment of HEK293T cells with Torin1, a new potent and selective mTORC1 inhibitor, reduced mTOR protein levels (Thoreen et al. 2009) suggesting that mTORC1 inhibition can influence the content of individual proteins in the mTORC1 pathway. Another interesting observation was down regulation of actin protein upon rapamycin treatment in the heart irrespective of

genotype. This was observed only in the heart but not in other tissues (see below). To ensure equal loading we also stained PVDF membranes with Coomassie blue following the Western. As seen in Fig. 4, even staining is observed for all of the lanes indicating equal loading. Down regulation of actin by rapamycin was also reported in cell lines such as U87 and LAPC-4 (Gera et al. 2004), as a shift of actin mRNA from well translated polysomes to poorly translated monosomes was observed. It was further shown that sensitivity of protein translation to rapamycin was dependent upon cyclin D1 and c-myc expression, which is determined by AKT activity. Therefore, reliability of actin as a loading control needs to be verified in the tissue and the cell line of interest when mTORC1 inhibition is investigated.

AMPK is a negative regulator of mTORC1, therefore AMPK protein and phosphorylation state (phosphorylated is active form) were determined in BCATm KO and wild-type mouse heart. Interestingly, compared to the wild type, AMPK phosphorylation was reduced in the KO heart indicating lower kinase activity of this enzyme. A similar pattern was observed for the ratio of phosphorylated form over total form. Lower active AMPK means that the inhibitory effect of AMPK on mTORC1, mediated through TSC2 phosphorylation, was reduced, consistent with mTORC1 over-activation and heart hypertrophy. Caspase-3 cleavage (activation), which is indicative of apoptosis, occurred at the same level for both the KO mice and the wild-type mice.

To identify protein molecules that might be involved in heart hypertrophy and the effect of rapamycin on these proteins, two-dimensional gel electrophoresis of the heart tissue extracts was performed. Preliminary proteomic analysis revealed up regulation of heat shock proteins including HSP27, HSP20, and GAPDH (Fig. 5). Western blot analysis of the heart samples confirmed HSP27 and GAPDH increased in the hearts of the female KO mice (Fig. 6). Rapamycin treatment normalized and increased HSP27 in the KO and WT mice, respectively. Unlike HSP27, rapamycin treatment reduced GAPDH levels in both the WT and KO mice. Finally, up regulation of HSP27 and GAPDH, which was observed in the heart samples of the KO mice, were not observed in the gastrocnemius, suggesting specificity of HSP27 and GAPDH up regulation to the heart (Fig. 6). Nonetheless, HSP27 protein level was reduced in both the heart and gastrocnemius tissues in the WT and KO mice upon rapamycin treatment suggesting similarity of pathways regulating HSP27 in these tissues. To verify equal loading, the membranes were stained following Western blot with Coomassie blue dye (Fig. 6a). As is seen in this figure the loading is even for all samples within each tissue.

Unlike heart, mTORC1 activation in the gastrocnemius of the BCATm KO mice was complex with S6 showing no increase but 4E-BP1 displaying a sixfold increase

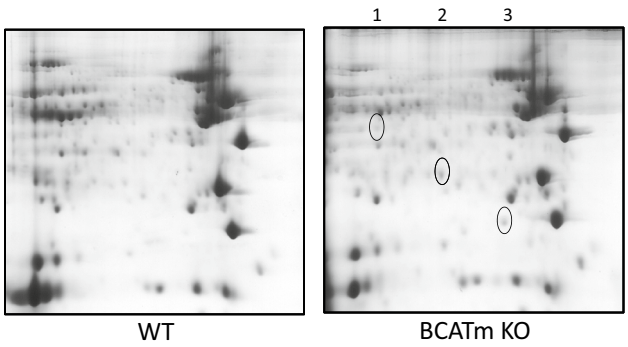

Fig. 5 Two-dimensional gel electrophoresis of the protein from heart muscle from the wild type (WT, *left*) and BCATm KO (*right*) mice on NBCAA diet. Spots were excised from the gel and identified using a TOF–TOF tandem mass spectrometer as described in "Materials and methods". *Spots #1*, *2* and *3* are HSP20, HSP27, and GAPDH, respectively. The *images* are representative of three 2D experiments per condition

in phosphorylation compared to the WT controls (Fig. 7a, b). The ratio of the phosphorylated to total protein followed a similar pattern as the phosphorylated from. Phosphorylation of S6 ribosomal protein enhances translation of mRNAs containing repressive 5-terminal oligopolypyrimidine (5′TOP) tracts (Jefferies et al. 1997) while phosphorylation of 4E-BP1 prevents its association with eIF4E thus allowing cap-dependent protein translation. We have not seen any report showing differential phosphorylation of mTORC1 substrates, although it has been suggested that mTOR substrates are treated differently by this kinase (Choo and Blenis 2009). This suggestion stems from the fact that in mouse embryonic stem cells, 4E-BP1 phosphorylation at Thr37/46 by mTORC1 is resistant to rapamycin inhibition, while the phosphorylation of S6K by mTORC1 is not (Choo et al. 2008; Thoreen et al. 2009). This could also explain why treatment with rapamycin did not reduce the phosphorylation level of 4E-BP1 in the gastrocnemius of the BCATm KO mice. P-AMPK which is decreased in the heart of the KO mice remained the same for all gastrocnemius samples. In addition, similar to heart, some caspase-3 cleavage was observed but appeared the same for all of the gastrocnemius samples irrespective of genotype or rapamycin treatment.

BCAA induced spleen enlargement in the BCATm KO mice (Fig. 3c). Enhanced phosphorylation of S6 and 4E-BP1 in the spleen samples of the KO mice was observed indicating activation of mTORC1 in this tissue (Fig. 8a, b). The ratios of phosphorylated forms of S6 and 4E-BP1 proteins to the total proteins were higher for the KO mice as compared to the WT controls and reduced upon rapamycin treatment. Similar to heart, phosphorylation of mTOR was found to be similar for both the wild type and KO mice in the spleen. Similar to gastrocnemius,

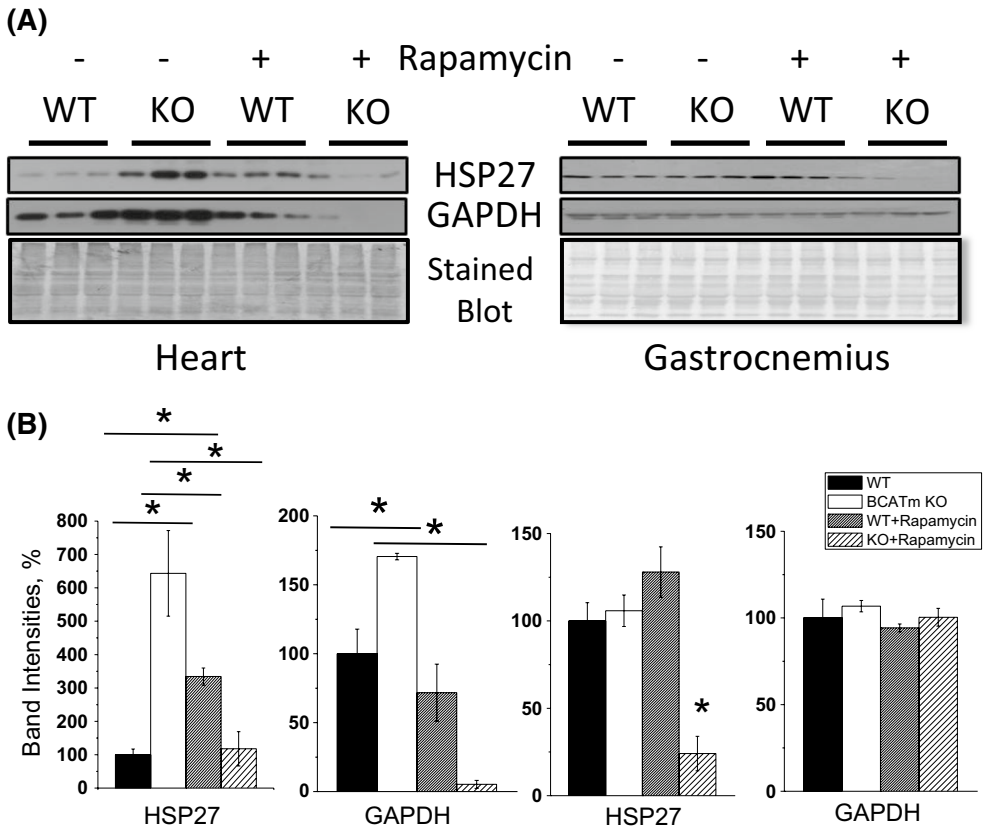

Fig. 6 Up regulation of HSP27 and GAPDH in the heart muscle but not skeletal muscle of BCATm KO mice. **a** Western blotting was used to determine the relative expression levels of these proteins. Gastrocnemius of the BCATm KO mice did not show any up regulation of these proteins compared to the WT controls. The PVDF membranes were stained with Coomassie blue to ensure equal loading. **a** Intensities of the bands were determined from the three samples in the Western blots by ImageJ software (**b**). Significant differences ($p \leq 0.05$) are marked by *asterisk*

total and phosphorylated AMPK protein remained similar for the WT and BCATO KO irrespective of the treatment. Inclusion of rapamycin in the diet inhibited mTORC1 pathway, which likely prevented splenomegaly, and is consistent with splenomegaly reversal by rapamycin reported previously (Cen and Longnecker 2011).

Compared to the WT controls, S6 protein phosphorylation in BCATm KO Liver was increased 2.6-fold and showed the highest level of phosphorylation of any BCATm KO tissue (Fig. 9a, b). Concomitantly, mTOR and 4E-BP1 proteins were highly phosphorylated. Although total mTOR and 4E-BP1 proteins were also increased by rapamycin treatment, only in the BCATm KO treated mice were differences significant ($p < 0.05$). Due to upregulation of total 4E-BP1 protein, the ratio of the phosphorylated to total protein was lower for rapamycin-treated KO mice as compared to untreated KO mice. With fourfold, the highest induction of mTOR phosphorylation at Ser2448 was observed in the liver of the BCATm KO mice compared to the WT controls while in other tissues, induction of mTOR phosphorylation varied from undetectable to twofold suggesting that mTOR activation in the liver is most responsive to increased BCAA concentrations (Fig. 9a, b). Liver was the only tissue showing statistically significant difference for the ratio of phosphorylated mTOR over total mTOR protein. Interestingly, however, in comparison to the WT control group, no liver hypertrophy was observed in the KO mice on NBCAA with sham capsules (Fig. 3a) despite the fact that mTORC1 of this tissue was the most responsive to elevated BCAAs. Surprisingly, the liver of BCATm KO mice on rapamycin diet also showed elevated mTORC1 activation as compared to the wild type treated with rapamycin or the regular diet albeit slightly reduced phosphorylation in the KO mice on rapamycin diet. Phosphorylation of AMPK was found to be higher in the 6-h fasted BCATm KO liver (Fig. 9a, b). Given the fact that AMPK activation causes mTORC1 inhibition in cell culture systems, it is not known why mTORC1 is still active. One possibility is that activation of AMPK is insufficient to counter mTORC1 activation by BCAAs.

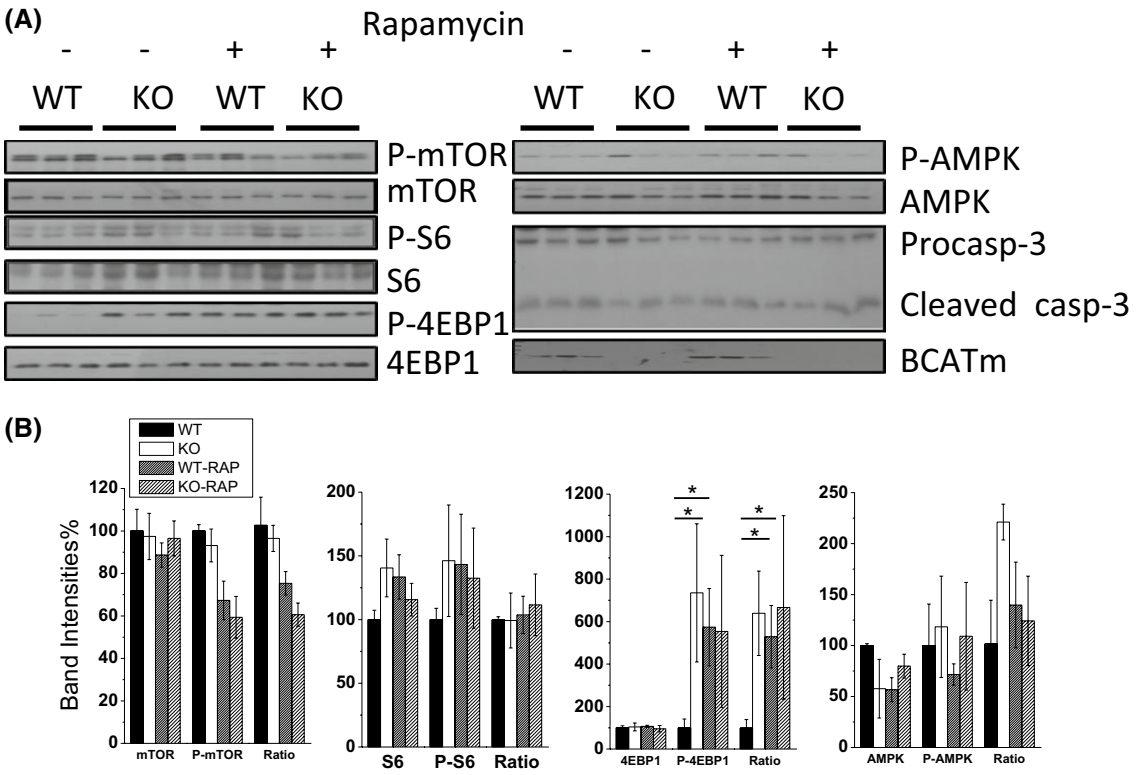

Fig. 7 Lack of mTORC1 activation in the gastrocnemius muscle from the wild type and BCATm KO mice. **a** Western blotting was used to determine the relative expression and phosphorylation levels of these proteins. **b** Quantification of the bands from the three samples in the Western blots by ImageJ for mTOR, S6, 4E-BP1, and AMPK, (**b**). Minimal cleavage of procaspase-3 (Procasp-3) was observed for all samples suggesting the presence of basal apoptosis in this tissue. Significant differences ($p \leq 0.05$) are marked by *asterisk*

Discussion

To our knowledge, this is the first report to study the chronic effect of BCAA on mTORC1 activation and organ size. We demonstrate that female BCATm KO mice have greatly increased plasma BCAA concentration, hypertrophy of heart, spleen, and kidneys. Unlike kidneys, hypertrophy of the heart and spleen appeared to be the result of amino acid mediated mTORC1 activation, because rapamycin treatment prevented these hypertrophies. Prevention of hypertrophy of the heart in BCATm KO mice agrees with the previous reports that inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload (McMullen et al. 2004; Gao et al. 2006). mTORC1 activation coincided with up regulation of a number of heat shock proteins. This can be explained by the fact that mTOR phosphorylates and activates heat shock transcription factor 1 (HSF1) which leads to overexpression of heat shock proteins including HSP27 (Chou et al. 2012). On the other hand, HSP27 binds to cytochrome C and prevents its interaction with Apaf1 blocking the intrinsic apoptosis pathway (Bruey et al. 2000). Therefore, it appears that HSPs are upregulated to prevent damage to the heart, which is in agreement with protective effects of HSPs in heart hypertrophy reported previously (Toko et al. 2008). In addition to HSPs, mTORC1 activation was accompanied by upregulation of the glycolytic enzyme, GAPDH, which is consistent with mTORC1 activation of glycolysis (Cairns et al. 2011). Enhanced glycolytic activity is a hallmark of heart hypertrophy due to elevated reliance on glucose as a fuel source for this tissue (Kolwicz and Tian 2011). Despite normalization of S6 and 4E-BP1 phosphorylation in rapamycin-treated BCATm KO mice, mTOR phosphorylation in the heart was reduced below the values observed for the control wild-type mice. This observation suggests that no linear relationship exists between mTORC1 phosphorylation at Ser 2448 and S6 activation and 4E-BP1 inactivation by mTORC1 complex (Lee et al. 2014; Chiu et al. 1994). Histological examination of the hypertrophied hearts in the male BCATm KO mice revealed the absence of any pathology in this tissue (Davoodi et al. unpublished data). Lack of tissue pathology combined with lack of difference in procaspase-3 cleavage (activation), which is indicative of apoptosis, in the KO mice as compared to the wild-type mice suggest

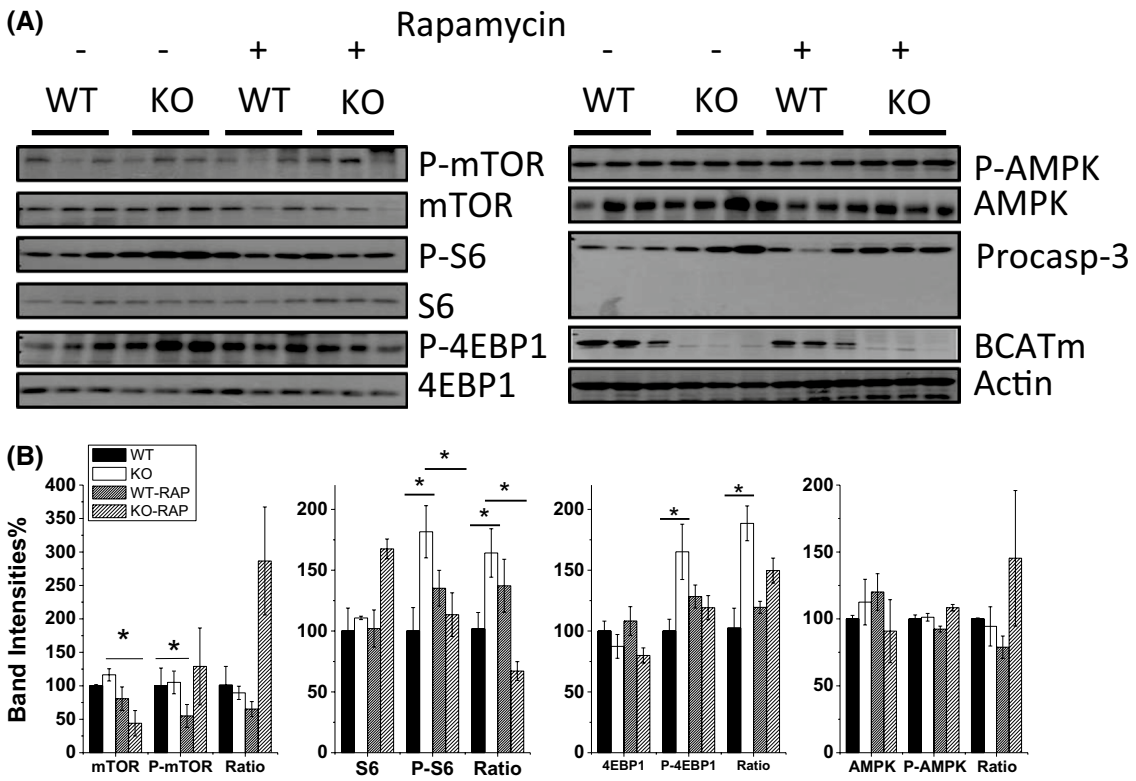

Fig. 8 Activation of mTORC1 signaling pathway in the spleen samples of BCATm KO mice. Tissue proteins were separated by SDS-PAGE electrophoresis and mTOR, S6, 4E-BP1, and AMPK were detected using Western blotting (**a**) and protein bands from the three samples in the Western blots were quantified using ImageJ (**b**). Absence of cleaved caspase-3 indicates the absence of apoptosis, although the procaspase-3 (Procasp-3) levels showed variation from one sample to another. Significant differences ($p \leq 0.05$) are marked by *asterisk*

the absence of pathological heart hypertrophy (Putinski et al. 2013).

Unlike the heart, the weight of the gastrocnemius muscle in the female KO mice was less than the wild-type mice. In this respect the female KO mice were different from the male mice, because the weight of the gastrocnemius in the male KOs remained the same as the WT mice. The absence of muscular hypertrophy in the KOs males was attributed to a protein futile cycle (She et al. 2007) by the original paper describing BCATm KO mice. According to this model, constitutive activation of mTOR increases protein synthesis which is compensated for by increased protein degradation creating a futile cycle that prevents skeletal muscle hypertrophy. Both measurements of 3-methylhistidine and the higher concentrations of BCAAs in starved BCATm KO mice compared to fed animals is consistent with this hypothesis. Our observation that the sustained treatment with rapamycin renders BCATm KO gastrocnemius muscles a normal size agrees with this hypothesis except that an imbalance between protein synthesis and degradation rate in the female KO mice must still exist making the gastrocnemius in KO females smaller than the WTs. Another possibility stems from the observation that leucine activated mTORC1 can phosphorylate IRS1 effectively blocking the stimulatory effect of insulin (Um et al. 2004). This possibility is ruled out, because a high phosphorylation level of IRS1 at Ser636, which is mediated by mTORC1, remained the same for all of the gastrocnemius samples from the WT and KO mice irrespective of the rapamycin treatment (data not shown). In addition, mTORC1 activation causes the activation of S6K which in turn blocks mTORC2 complex. Inhibition of mTORC2 in turn, negatively impacts PKCα-mediated myogenesis (Ge and Chen 2012). Loss of muscle mass due to apoptosis, which has previously been suggested (Wang et al. 2006), is unlikely, because caspases-3 activation (cleavage) in the KO mice occurred to the same extent as in the WT mice. Female BCATm KO mice had reduced movement compared to female WT mice and, by inference, decreased muscle use. This might be another explanation for the reduced skeletal muscle size in the female BCATm KO mice compared to the wild-type controls.

4E-BP1 phosphorylation in the rapamycin-treated gastrocnemius samples of the BCATm KO mice failed to

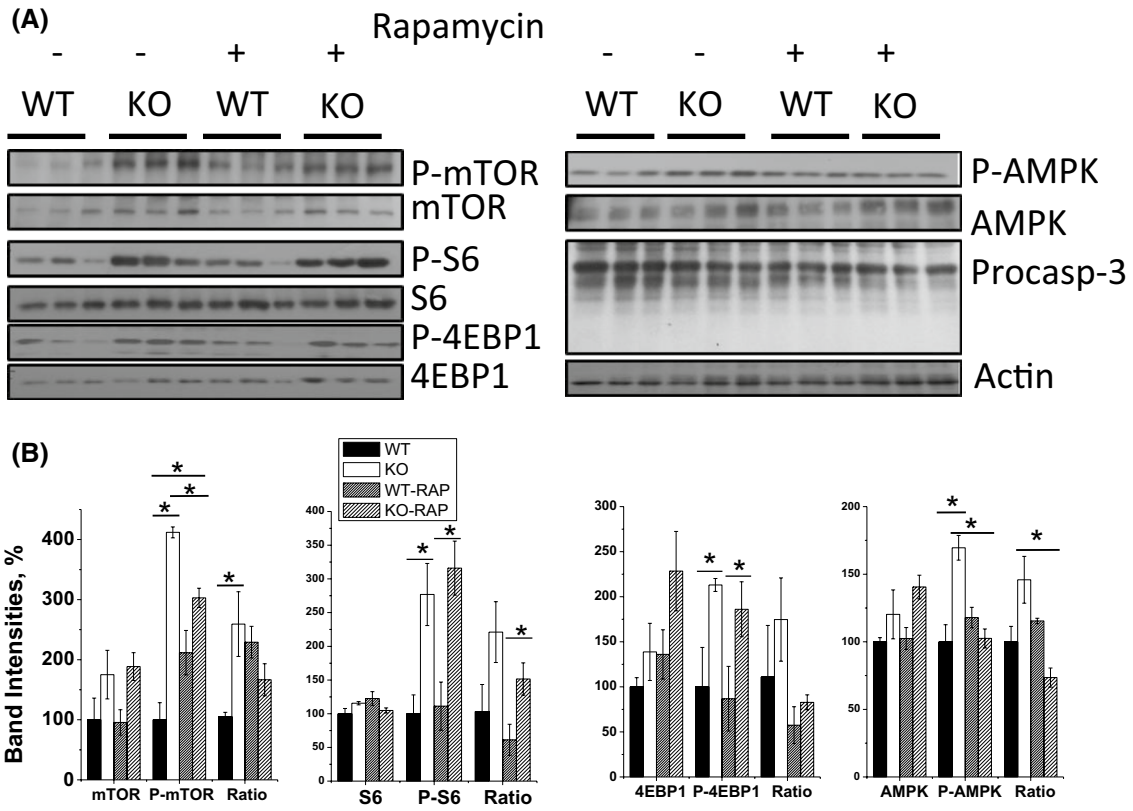

Fig. 9 Activation of mTORC1 signaling pathway of the liver samples in the BCATm KO mice determined by Western blot, **a**, and quantification of the bands from the three samples in the Western blots by ImageJ for mTOR, S6, 4E-BP1, and AMPK, **b**. The Western blot for BCATm is not shown for the liver because this protein is not expressed in rodent liver (Hutson et al. 1992). Apoptosis was not seen in liver because cleaved caspase-3 was not present. Significant differences ($p \leq 0.05$) are marked by *asterisk*

show any decrease in its phosphorylation when compared to untreated KO mice. Rapamycin forms a complex with FKBP12 (also known as FKBP1a) which then binds to mTORC1 inhibiting its activity (Chiu et al. 1994). The mechanism of inhibition is through the restriction of the substrate entry into the active site of the enzyme (Yang et al. 2013). It seems that rapamycin's ability to inhibit mTORC1 is dependent upon the affinity of the substrate to the kinase and thus determined by the substrate sequence (Shimobayashi and Hall 2014). Therefore, it can be suggested that the affinity of mTORC1 for 4E-BP1 phosphorylation supersedes that of S6K. A surprising observation of the current study was a dramatic increase in phosphorylation of 4E-BP1 in the gastrocnemius samples of the rapamycin-treated wild-type mice as compared to untreated wild-type controls. It was very recently shown that at lower doses, rapamycin increases mTORC1 activity in skeletal muscle (Lee et al. 2014). This was explained by rapamycin's ability to hinder FKBP12 inhibition of Ryanodine receptor 1 (RyR1) causing enhanced calcium release from sarcoplasmic reticulum, mTORC1 activation, and improved muscle function. Improved movement of the rapamycin-treated wild type and BCATm KO mice is consistent with this observation, and implies that the systemic concentrations of rapamycin in these two studies were more or less similar. Because, the drug was delivered through injection in the study by Lee et al. (2014) while we used diet to maintain a low yet consistent presence of the drug in the animal's system, a direct comparison of the drug doses for these two studies is not possible. Increased phosphorylation of 4E-BP1 in rapamycin-treated gastrocnemius muscles of the rapamycin fed wild type and BCATm KO mice may be interpreted based on antagonizing effect of rapamycin on FKBP12–RyR1 interaction rather than inhibition of mTORC1 by FKBP12–rapamycin complex. Based on our data and in light of recent discoveries sited above it is tempting to suggest that the outcome of rapamycin effect on a tissue is dependent upon intracellular concentration of rapamycin and its receptors, FKBP proteins that is, as well as the effect of rapamycin on other proteins like TGF-beta type I serine/threonine kinase receptor (Wang et al. 1994), and Ryanodine receptors that interact with FKBP proteins.

Phosphorylation of mTOR was found to be similar for both the WT and KO mice in the spleen despite the activation

of this pathway in the BCATm KOs as reflected by enhanced phosphorylation of mTORC1 substrates. This might be due to the fact that seemingly a small change in mTORC1 activation might cause dramatic up regulation of its downstream signaling targets. Inclusion of rapamycin in the diet inhibited the mTORC1 pathway, which likely prevented splenomegaly, and is consistent with splenomegaly reversal by rapamycin reported previously (Cen and Longnecker 2011). Unlike spleen, BCATm KO liver, which had the highest level of mTORC1 activation, maintained a normal size indicating that mTORC1 activation alone is insufficient to induce the hypertrophy of this organ and other pathways are also involved. This is consistent with the observation that induction of mTORC1 pathway by oral administration of leucine induces mTORC1 activity in liver without elevation of global protein synthesis (Anthony et al. 2001). Surprisingly, rapamycin-treated BCATm KO mice displayed liver hypertrophy, which coincided with the inability of rapamycin to inhibit mTORC1-mediated phosphorylation of 4E-BP1 and S6 proteins. This is similar to a previous observation showing that treatment of pregnant mice with rapamycin causes a significant increase in hepatocyte proliferation rate (Gielchinsky et al. 2010). The authors attributed this phenomenon to activation of mTORC1 combined with rapamycin treatment. In our case, mTORC1 was activated by elevated BCAAs with rapamycin unable to block downstream mTORC1 pathway of S6K and 4E-BP1. Given the fact that rapamycin cannot block all mTORC1 functions (Liu et al. 2010; Thoreen et al. 2009) specially under conditions used in our study, it seems that partial inhibition of mTORC1 combined with S6K activation and 4E-BP1 inactivation can cause increased rate of cell proliferation and liver hypertrophy (Liu et al. 2010; Thoreen et al. 2009; Gielchinsky et al. 2010), although one should not ignore the extent of mTORC1 activation and the dose of inhibitor.

Epididymal fat remained the same for the WT and KO mice which is in agreement with MRI data. Finally, considerable variation was observed for the effect of rapamycin on total proteins of the mTOR pathway and their phosphorylation state in different tissues, suggesting complexity of regulation of proteins involved in mTOR pathway. In addition, elevated plasma BCAA did not cause ubiquitous activation of mTOR suggesting tissue-specific regulation of mTOR by BCAAs.

Similar to the male BCATm KO mice (She et al. 2007), plasma BCAA concentrations were greatly increased in the KO female mice compared to WT controls. Because large neutral amino acids use the same transporter as leucine, increased BCAA concentrations create competition for cellular entry (Segawa et al. 1999; Yudkoff et al. 2005) which could explain, at least in part, increased concentration of Trp, Met, and Thr in the plasma. Tyr, which uses the same transporter as other large neutral amino acids, was not increased suggesting that in addition to competition for transport other factors like metabolism of these amino acids are also involved. Unlike above-mentioned amino acids, Ala was reduced in plasma which is consistent with the results obtained for the male mice (She et al. 2007). This can be explained by the fact that BCAAs provide nitrogen for skeletal muscle alanine synthesis (Haymond and Miles 1982). Therefore, reduced concentrations of plasma Ala are likely due to the block in transamination of BCAAs by BCATm (Gray et al. 2007).

RER values for the WT and BCATm KO mice did not exceed one, consistent with lack of body fat accumulation in the WT and the KOs. In addition, RER and energy expenditure were similar for the wild type and KO mice on NBCAA diet containing either sham capsules or encapsulated rapamycin up to the 7th day of the treatment. However, the numbers for the BCATm KO mice on sham capsules were gradually decreased such that on the 9th day of the treatment they were statistically different. Inclusion of rapamycin prevented the reduction in the KO mice suggesting that high BCAA concentrations act through mTOR to reduce RER and energy expenditure. It is highly likely that most of the effects of constitutively elevated BCAA concentrations in plasma and tissue are mediated through the mTORC1 pathway, which can be countered by rapamycin resulting in a more normal phenotype. A previous report (Lynch et al. 2002), which used drinking water to deliver and increase plasma leucine, measured the effect of leucine on protein synthesis in rats; however, the study did not report changes in organ size.

In summary, we demonstrate that chronically elevated plasma leucine can cause organ hypertrophy in a selective manner mediated through the activation of mTORC1 pathway. In addition, elevated BCAA results in hypertrophy of the heart and spleen, which can be blocked by rapamycin. Given that the concentration of BCAAs in the plasma can be easily controlled by adjusting BCAA content of the diet, and considering that the elevated BCAA activates mTORC1, BCATm KO mice provide a useful model to study processes regulated by mTOR-like organ hypertrophy, insulin resistance (Dann et al. 2007), T-cell activation (Ananieva et al. 2014; Lo et al. 2014), autophagy and aging (Martin et al. 2014).

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Conflict of interest There is no conflict of interest to report.

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