Amino Acids (2012) 43:725–734
DOI 10.1007/s00726-011-1123-8

ORIGINAL ARTICLE

# Isoleucine or valine deprivation stimulates fat loss via increasing energy expenditure and regulating lipid metabolism in WAT

**Ying Du · Qingshu Meng · Qian Zhang · Feifan Guo**

Received: 17 May 2011 / Accepted: 7 October 2011 / Published online: 21 October 2011


**Abstract** There has been a growing interest in controlling body weight by increasing dietary levels of leucine recently. By contrast, we have focused on studying the effect of deficiency of branched-chain amino acids (BCAAs) leucine on lipid metabolism. We previously have shown that mice fed a leucine-deficient diet for 7 days exhibit significant changes in lipid metabolism as demonstrated by suppressed lipogenesis in the liver and increased fat mobilization in white adipose tissue, the latter of which was found to be caused by increased lipolysis in WAT and uncoupling protein 1 expression in brown adipose tissue. The goal of our current study is to investigate whether the above effects of leucine deficiency can be generalized to the deficiency of other BCAAs including valine and isoleucine. In our current study, we show that valine or isoleucine deficiency has similar effects on reducing fat mass to leucine deprivation, in a similar manner as those observed during leucine deprivation.

**Keywords** Isoleucine · Valine · Deprivation · Fat loss

## Introduction

Obesity and its associated diseases including type 2 diabetes and fatty livers are serious global problems that greatly threaten human health. A common pathological change leading to these metabolic diseases is a disorder in lipid metabolism, which includes increased synthesis and decreased utilization of fatty acids in tissues such as liver, white adipose tissue (WAT) and brown adipose tissue (BAT) (Fujioka et al. 1987; Marchesini et al. 2001; Trayhurn 1979). Though extensive studies have been conducted concerning different aspects of metabolic diseases, molecular mechanisms underlying are still not fully understood and effective treatments are still missing.

Amino acids serve as precursors in protein synthesis (Loftfield and Harris 1956). In addition, they are important signaling regulators (Kimball and Jefferson 2006). Branched-chain amino acids (BCAAs) are the most noticed essential amino acids that have non-linear aliphatic side-chains, which include leucine, isoleucine and valine. Recent studies have shown that increasing intake of BCAAs has significant impact on lipid and glucose metabolism (Layman and Walker 2006; Doi et al. 2005). The effect of increasing dietary BCAAs content, however, is controversial. Some studies show that increased BCAAs prevent high-fat diet-induced obesity (Zhang et al. 2007; Nishimura et al. 2010), whereas others report that it either has no effect or leads to insulin resistance (Nairizi et al. 2009).

By contrast, we have focused on studying the effect of deficiency of BCAAs, for example leucine, on lipid metabolism. We unexpectedly discovered that mice fed a leucine-deficient diet for 7 days exhibit significant changes in lipid metabolism as demonstrated by suppressed lipogenesis in the liver (Guo and Cavener 2007) and increased fat mobilization in WAT (Cheng et al. 2010), the latter of which was found to be caused by increased lipolysis in WAT and uncoupling protein 1 (UCP1) expression in BAT (Cheng et al. 2010). However, it remains unclear if deficiency of other BCAA, isoleucine and valine, would have

Y. Du · Q. Meng · Q. Zhang · F. Guo (✉)
Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, The Graduate School of the Chinese Academy of Sciences, 294 Taiyuan Road, 200031 Shanghai, China
e-mail: ffguo@sibs.ac.cn

similar effects as leucine. A previous study showed that levels of fatty acid synthase (*Fas*) mRNA are suppressed in HepG2 cells in medium deficient of any essential amino acid, but not by non-essential amino acids (Dudek and Semenkovich 1995), suggesting that deficiency of isoleucine or valine may produce similar effects on fat loss and suppression of lipogenesis in the livers. The goal of present study is to investigate this possibility by measuring various metabolic parameters and examining changes in lipid metabolism in the liver, WAT and BAT.

## Methods

### Animals and diets

Wild-type male C57BL/6J mice were obtained from Shanghai Laboratory Animal Co., Ltd. (SLAC, Shanghai, China). Eight- to ten-week-old mice were kept on a 12-h light/dark cycle at 25°C and were provided free access to commercial rodent chow and tap water before experiments. Control (nutritionally complete amino acid), (−) Ile (isoleucine-deficient) and (−) Val (valine-deficient) diets were obtained from Research Diets, Inc. (New Brunswick, NJ). All diets were isocaloric and compositionally the same in terms of carbohydrate and lipid component. At the start of the feeding experiment, mice were acclimated to a control diet for 7 days and then randomly divided into either control, (−) Ile or (−) Val diet group with free access to either control, (−) Ile, or (−) Val diet, respectively, for 7 days, a duration that is comparable to experiments performed with leucine deprivation (Guo and Cavener 2007; Cheng et al. 2010). Food intake and body weight were monitored daily. Before mice were sacrificed by $CO_2$ inhalation, the total body fat content of each mouse was quantified in vivo by a mini-spec nuclear magnetic resonance (NMR) spectrometer (Bruker Corp.), according to manufacture's protocol. Liver, WAT and BAT weight was recorded at the time of sacrifice. Tissues were isolated and snap-frozen and stored at −80°C for future analysis. These experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Sibs, CAS.

### Indirect calorimetry

After 6 days feeding with either control, (−) Ile, or (−) Val diet, mice were maintained in a comprehensive lab animal monitoring system (Columbus Instruments, Columbus, OH) for 24 h according to the instructions of the manufacturer. Volume of $O_2$ consumption and $CO_2$ production was continuously recorded over 24-h period.

### Rectal temperature measurement

The rectal temperatures of the mice were measured using a rectal probe attached to a digital thermometer (Physitemp. Inc., NJ, USA).

### Serum measurements

Serum was obtained by centrifugation of clotted blood and then stored at −80°C. Blood glucose levels were measured by a Glucometer Elite monitor (Medisense). Serum triglyceride (TG), cholesterol, free fatty acids (FFA) and glycerol were determined using triglyceride kit (BTKH Clinical Reagents, Beijing, China), cholesterol kit (BTKH Clinical Reagents, Beijing, China), NEFA kit (Jiancheng Biotechnology, Nanjing, China) and Glycerol Assay kit (SinoPCR, China), respectively. All of these assays were performed according to manufacturer's instructions.

### Western blot analysis

Whole-cell lysates from frozen tissues were isolated using RIPA lysis buffer (150 mM Tris–HCl, 50 mM NaCl, 1% NP-40, 0.1% tween-20). Protease and phosphatase inhibitors were added to all buffers before experiments. Western blot was performed as previously described (Cheng et al. 2010). Protein concentrations were assayed with BCA Kit (Pierce). Primary antibodies [anti-FAS antibody (BD Scientific), anti-p-HSL, anti-p-PKA substrate antibodies (Cell Signaling), and anti-actin antibody (Sigma), anti-SREBP1c and anti-UCP1 antibodies (Santa Cruz Biotechnology)] were incubated overnight at 4°C and specific proteins were visualized by ECL Plus (Amersham Biosciences). Band intensities were measured using Quantity One (Bio-Rad Laboratories) and normalized to actin or non-specific proteins.

### RNA isolation and relative quantitative RT-PCR

RT-PCRs were performed as described previously (Cheng et al. 2010). Total RNA was prepared from frozen tissues with TRIZOL (Invitrogen) reagent. One microgram of RNA was reverse transcribed with PrimeScript RT reagent kit (Takara). Quantitative amplification by PCR was carried out using SYBR Green I Master Mix reagent by ABI 7500 system (Applied Biosystem). PCR products were subjected to a melting curve analysis. Cycle numbers of both GAPDH (as an internal control) and cDNAs of interest at a specific threshold within the exponential amplification range were used to calculate relative expression levels of the genes of interest. The sequences of primers used in this study are available upon request.

### Statistics

All data are expressed as mean ± SEM. Significant differences between (−) Ile or (−) Val and control group were assessed using the two-tail student $t$ test. $p < 0.05$ was considered statistically significant.

## Results

### Isoleucine or valine deprivation significantly decreases body and fat weight

We previously have shown that mice maintained on a leucine-deficient diet undergo rapid loss of abdominal fat (Cheng et al. 2010). The goal of present study is to investigate whether isoleucine or valine deficiency has similar effects on mice. For this purpose, mice were fed control, isoleucine-deficient or valine-deficient diets for 7 days, a period that is comparable to those we examined during leucine deprivation (Cheng et al. 2010). Isoleucine or valine deprivation for 7 days resulted in about 24 or 16% reduction in body weight compared with mice maintained on a control diet (Fig. 1a). Consistent with those observed during leucine deprivation (Cheng et al. 2010), we found that the weights of tissue mass, including liver, epididymal fat and brown fat, were also significantly decreased (Fig. 1b). Furthermore, the total body fat was significantly decreased by isoleucine or valine deprivation in comparison with the control mice, while there was a little increase in the proportion of lean mass in isoleucine- or valine-deprived groups as measured by NMR (Fig. 1c), though the amount of lean mass was decreased (Fig. 1d). Therefore, the decreased body weight is caused by the reduced fat and lean mass, as well as the decreased amount of body fluid (Fig. 1d).

Similar to those observed during leucine deprivation, we found that serum levels of triglyceride (TG), cholesterol, free fatty acid (FFA) and glycerol were lower in isoleucine or valine-deprived mice compared with those maintained on a control diet (Table 1).

### Isoleucine or valine deprivation decreases food intake and increases energy expenditure

As decreased fat mass is caused by decreased food intake and/or increased energy expenditure, we compared food intake among different groups. In contrast to the 15% reduction in food intake by leucine deprivation (Cheng et al. 2010), isoleucine or valine deprivation has much more profound impact on food intake reduction (39% for isoleucine and 25% for valine), compared with mice maintained on a control diet (Fig. 1e). A pair-fed (pf) group was included in our preliminary experiments to control for the reduction in daily food intake in (−) isoleucine or valine group. Mice in the pf groups were provided with 39 or 25% less food compared to mice in the control diet group (Fig. 1e). Pf groups for isoleucine and valine decreased body weight 16 and 10%, respectively, compared with mice maintained on a control diet (Fig. 1f). In every case, mice maintained on an isoleucine or valine-deficient diet lost more weight compared with those in control or pf group, suggesting a specific effect of isoleucine or valine deprivation on body weight reduction.

We previously showed that mice maintained on leucine-deficient diet exhibit increased energy expenditure (Cheng et al. 2010). Therefore, we measured energy expenditure by indirect calorimetry in mice under control, isoleucine-deficient or valine-deficient diets for 7 days. The total energy expenditure (24-h $O_2$ consumption, normalized to lean body mass) was markedly increased in isoleucine or valine-deprived mice, compared with mice maintained on a control diet (Fig. 2a). The respiratory exchange ratio (RER, $VCO_2/VO_2$) was also lower in isoleucine or valine-deprived mice during both dark and light phases (Fig. 2b). Consistent with those observations obtained during leucine deprivation (Cheng et al. 2010), we found that rectal temperatures measured at 3 p.m. in the afternoon (basal metabolic state) were significantly higher in isoleucine-deprived mice, compared with mice maintained on a control diet (Fig. 2c). By contrast, rectal temperature was significantly lower in valine-deprived mice (Fig. 2c). Different from those observed during leucine deprivation, the physical activity was significantly increased in isoleucine-deprived mice as measured in a metabolic cage (Fig. 2d). Similar to leucine, valine has no effect on physical activity (Fig. 2d).

### Isoleucine or valine deprivation regulates genes and proteins related to lipolysis, $\beta$-oxidation and lipogenesis in WAT

To investigate whether isoleucine or valine deprivation has similar regulatory effects on lipid metabolism in WAT as leucine deprivation, we examined changes in genes and proteins related to lipolysis, $\beta$-oxidation and lipogenesis in WAT of mice maintained on diet deficient for isoleucine or valine. mRNA levels of adipose triglyceride lipase (*Atgl*), the first enzyme regulating lipolysis (Schoenborn et al. 2006), were significantly increased by either isoleucine or valine deprivation compared with those maintained on a control diet (Fig. 3a). Levels of phosphorylated hormone sensitive lipase (HSL), the second enzyme regulating lipolysis (Greenberg et al. 2001), were significantly increased in WAT of isoleucine or valine-deprived mice compared with mice fed a control diet (Fig. 3b). Consistent

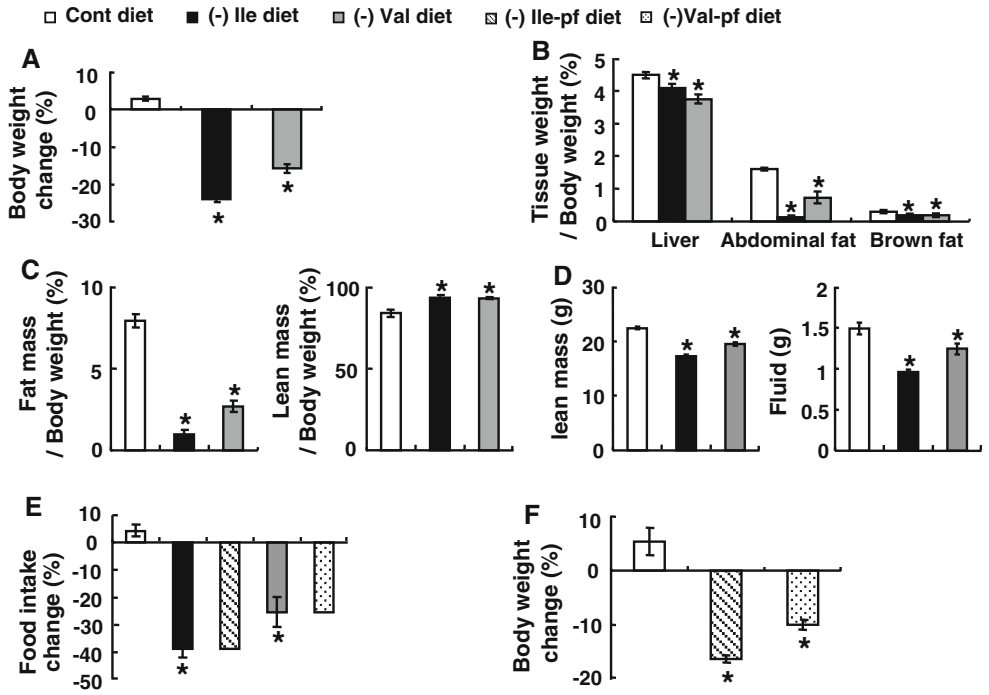


**Fig. 1** Body and fat weight are decreased in isoleucine- and valine-deprived mice. Mice were fed a control, isoleucine-, or valine-deficient diet for 7 days. Pf group for isoleucine or valine group, (−) Ile-pf and (−) Val-pf, respectively, was also included. Data are mean ± SEM of at least two independent experiments with mice of each diet for each experiment (control diet [$n = 6$]; (−) Ile diet [$n = 6$]; (−) Ile-pf diet [$n = 6$]; (−) Val diet [$n = 6$]; (−) Val-pf diet [$n = 6$]). Statistical significance was determined by the two-tail student $t$ test for the effect of either (−) Ile, (−) Ile-pf, (−) Val (−) Val-pf diet versus control diet (*$p < 0.05$). **a** Body weight reduction, **b** tissue weight (liver, WAT and BAT) in proportion to body weight, **c** body composition measured with NMR, **d** lean mass and body fluid, **e** food intake change, **f** body weight reduction of pair-fed groups

**Table 1** Serum measurements in mice maintained on different diets

| | Control | (−) Ile (mmol/L) | (−) Val |
|---|---|---|---|
| Glucose | 10.23 ± 0.57 | 7.72 ± 0.46* | 9.07 ± 0.63 |
| TG | 0.32 ± 0.02 | 0.17 ± 0.05* | 0.07 ± 0.01* |
| Cholesterol | 2.15 ± 0.05 | 1.29 ± 0.08* | 1.31 ± 0.05* |
| FFA | 0.49 ± 0.10 | 0.16 ± 0.02* | 0.21 ± 0.05* |
| Glycerol | 0.162 ± 0.019 | 0.075 ± 0.016* | 0.093 ± 0.010* |

Two- to three-month-old mice were fed a control-, isoleucine-, or valine-deficient diet for 7 days. Number of mice used: $n = 6$ in each group. Values represent data mean ± SEM. Statistical significance was determined by the two-tail student $t$ test for the effect of either (−) Ile or (−) Val diet *versus* control diet (*$p < 0.05$)

with increased levels of phosphorylated HSL, levels of phosphorylated substrate for PKA, the kinase that phosphorylates HSL (Watt et al. 2006), were also elevated in WAT of isoleucine or valine-deprived mice (Fig. 3b).

It has been shown that $\beta$-oxidation is regulated by the transcription factor peroxisome proliferators-activated receptor (*Ppar*)α, and its downstream target gene carnitine palmitoyltransferase 1 (*Cpt1*) (Ferre 2004; Lee et al. 2002), expression of which has been shown to be increased by leucine deprivation (Cheng et al. 2010). Consistent with these results (Cheng et al. 2010), we found that the expression levels of *Pparα* were significantly increased in WAT of mice maintained on an isoleucine-deficient diet compared with mice maintained on a control diet (Fig. 3c). Levels of *Cpt1* mRNA also show increased tendency in these mice (Fig. 3c). Expression levels of both *Pparα* and *Cpt1*, however, were significantly increased in WAT of mice maintained on a valine-deficient diet compared with those maintained on a control diet (Fig. 3c).

Consistent with effects of leucine deprivation (Cheng et al. 2010), levels of the critical enzyme for lipogenesis *Fas* mRNA (Nogalska et al. 2005), and its upstream regulator sterol regulatory element-binding protein (SREBP)-1c protein (Nogalska et al. 2005), were also greatly reduced in WAT of mice maintained on a isoleucine or valine-deficient diet compared with mice maintained on a control diet (Fig. 3d).

To investigate whether autophagy is involved in isoleucine or valine-induced fat loss, we examined the mRNA levels of genes encoding autophagy-related proteins including *ATG4a*, *ATG5*, *ATG7*, *ATG8/LC3* (Scherz-Shouval et al. 2007; Noda et al. 2010; Komatsu et al. 2005; Yousefi et al. 2006). All of these genes were up-regulated in WAT of mice maintained on an isoleucine or valine-deficient

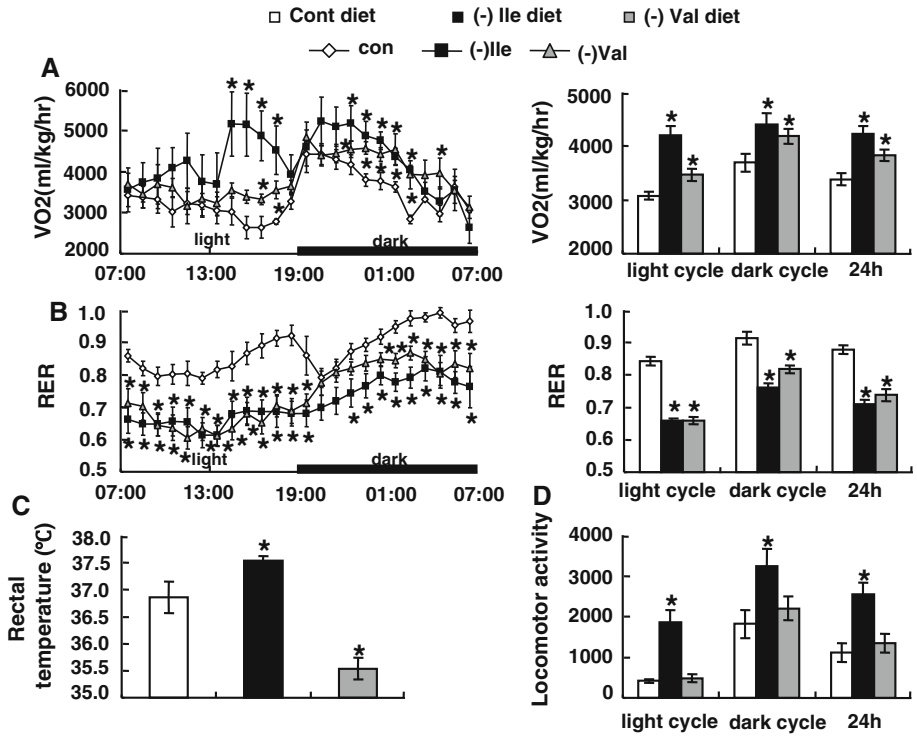


**Fig. 2** Oxygen consumption is increased in isoleucine- and valine-deprived mice. The energy expenditure was measured by indirect calorimetry in mice fed a control, isoleucine-, or valine-deficient diet for 7 days. Data are mean ± SEM of at least two independent experiments with mice of each diet for each experiment (control diet [$n = 6$]; (−) Ile diet [$n = 6$]; (−) Val diet [$n = 6$]) over 24–48 h after 6 h acclimation to the metabolic chamber. Statistical significance was determined by the two-tail student t test for the effect of either (−) Ile or (−) Val diet *versus* control diet (*$p < 0.05$). **a** 24-h Oxygen consumption, **b** respiratory exchange ratio (RER), **c** rectal temperature, **d** physical activity

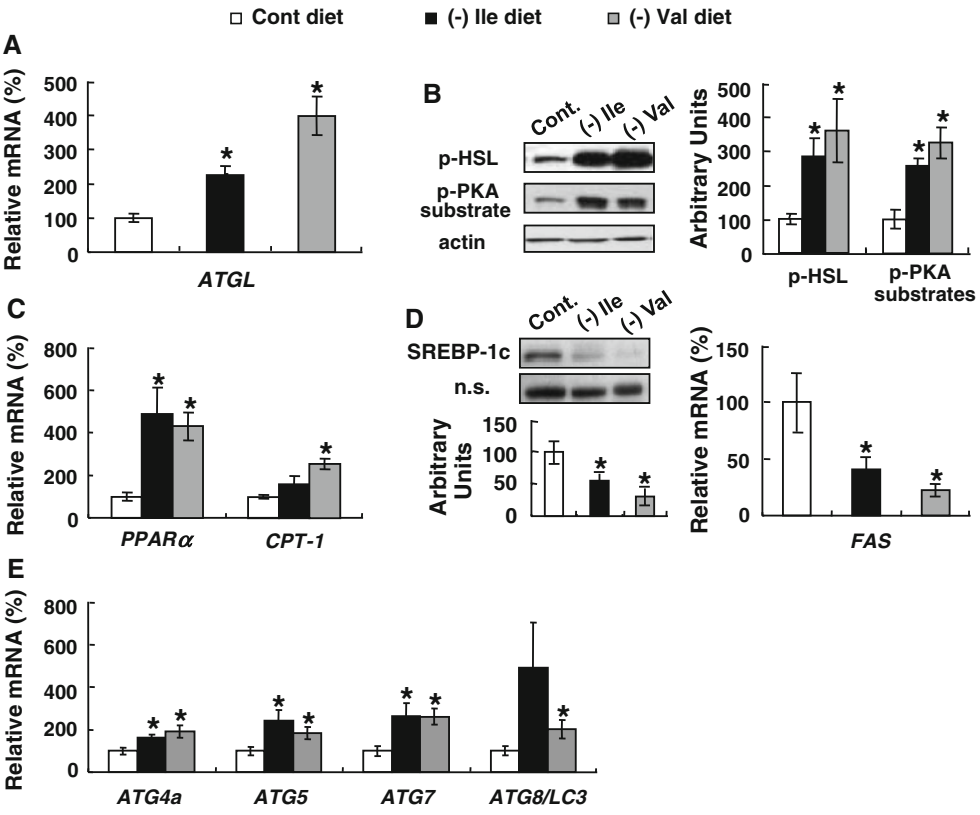


**Fig. 3** Changes of genes and proteins related to lipogenesis, *β*-oxidation, lipolysis in WAT of isoleucine- and valine-deprived mice. Mice were fed a control, isoleucine-, or valine-deficient diet for 7 days. Data are mean ± SEM of at least two independent real-time PCR experiments (**a**, **c**) or western blot (**b**, **d**) with mice of each diet for each experiment (control diet [$n = 6$]; (−) Ile diet [$n = 6$]; (−) Val diet [$n = 6$]). Statistical significance was determined by the two-tail student *t* test for the effect of either (−) Ile or (−) Val diet *versus* control diet (*$p < 0.05$). **a** *Atgl* mRNA, **b** p-HSL and p-PKA substrate proteins (*left*, western blot; *right*, p-HSL and p-PKA substrate proteins relative to actin), **c** *Pparα* and *Cpt-1* mRNA, **d** SREBP-1c protein (*top* western blot, *bottom* SREBP-1c protein relative to non-specific band) and *Fas* mRNA, **e** *Atg4a, Atg5,Atg7* and *Atg8/Lc3* mRNA

diet compared with mice maintained on a control diet (Fig. 3e).

### Isoleucine or valine deprivation increases UCP1 expression in BAT

Lipolysis is increased in isoleucine or valine-deprived mice compared with control groups. Increased lipolysis is normally accompanied by increased levels of serum TG and FFA. Serum levels of TG and FFA, however, were decreased in these mice compared with control groups, as shown in our current work (see Table 1). Our observation that serum TG and FFA levels are decreased in isoleucine or valine-deprived mice could be due to increased $\beta$-oxidation in WAT (Fig. 3c), and/or the FFA released from WAT is going to other tissues, including BAT and muscle as observed in leucine-deprived mice (Cheng et al. 2010). We therefore examined the changes of lipid metabolism related genes in BAT.

The main function of BAT is for thermogenesis, which is mediated by upregulation of UCP1 (Matthias et al. 2000). Consistent with our previous results during leucine deprivation (Cheng et al. 2010), isoleucine deprivation also increases levels of *Ucp1* mRNA and protein in BAT compared with mice maintained on a control diet (Fig. 4a, b). mRNA levels of peroxisome proliferators-activated receptor gamma coactivator (*Pgc*)*1*$\alpha$, which regulates the expression of UCP1 (Handschin and Spiegelman 2006), were also increased in the BAT of these mice (Fig. 4a, b). Similar results were obtained in mice under valine deprivation (Fig. 4a, b).

### Isoleucine or valine deprivation supresses FAS expression in the livers

We previously have shown that genes and proteins related to lipogenesis were significantly decreased in the livers of leucine-deprived mice (Guo and Cavener 2007), therefore, we examined expression of FAS in the livers of mice under isoleucine or valine deprivation. Similar to those observed during leucine deprivation (Guo and Cavener 2007), we found that *Fas* mRNA and protein levels were significantly decreased in the livers of mice under isoleucine or valine deprivation compared with those maintained on a control diet (Fig. 5a, b).

## Discussion

In our current study, we observed that isoleucine or valine deficiency has similar effects with leucine deprivation, including increases in lipolysis and expression of $\beta$-oxidation genes, and decreases in expression of lipogenic genes in WAT. In addition, UCP1 expression was also increased in BAT, which is consistent with increased oxygen consumption as measured by metabolic cages. Furthermore, valine or isoleucine deficiency also suppresses lipogenesis in liver. Taken together, BCAAs may regulate lipid metabolism via common mechanisms.

Isoleucine is an isomer of leucine. It has been shown that isoleucine supplement could prevent the tissue triglycerides accumulation (Nishimura et al. 2010) and balance blood glucose (Ikehara et al. 2008). For example, gavage administration of isoleucine has been shown to decrease the accumulation of tissue triglyceride via increasing expression of PPAR$\alpha$ and UCPs (Nishimura et al. 2010). Moreover, oral administration of isoleucine significantly decreased the plasma glucose concentration via stimulating glucose uptake in skeletal muscle (Doi et al. 2005), in addition to decrease gluconeogenesis in liver (Doi et al. 2007). By contrast, deficiency of isoleucine has been shown to synchronize growth of cells in G1 phase (Jakesz et al. 1984; Wynford-Thomas et al. 1985) and declines the sterol and fatty acid de novo synthesis in cells (Maltese et al. 1981). The effect of isoleucine deficiency on mice, however, is much less unknown. In current study, we show that, similar to those observed during leucine deprivation, isoleucine deficiency also significantly reduces body weight and abdominal adipose mass, which is further confirmed by much decreased fat content measured by NMR. In contrast to the effect of leucine deprivation, isoleucine deprivation increases slightly the proportion of lean mass, which could also be due to the significant reduction of fat mass in isoleucine-deprived mice. Consistent with the effect of leucine deprivation, isoleucine also significantly decreases food intake and increases energy expenditure. Furthermore, isoleucine deprivation results in similar changes in lipid metabolism in WAT, BAT and liver as those observed during leucine deprivation (Guo and Cavener 2007; Cheng et al. 2010).

The extent for the fat loss, however, is much more significant by isoleucine deficiency compared with leucine deprivation. One reason is because of its much more significant inhibitory effect on food intake (39% reduction) compared with leucine deprivation (15% reduction). The different extent in decreasing food intake, however, suggests a differential regulation on activities of orexigenic neurons that express agouti-related peptide (AgRP) and neuropeptide Y (NPY), and anorexigenic neurons that express proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) in the hypothalamus (Meister 2007; Cota et al. 2006; Lopez et al. 2010), by isoleucine compared with leucine deprivation.

Another reason may come from the differences in increased energy expenditure between isoleucine and leucine deficiency. Though both isoleucine and leucine

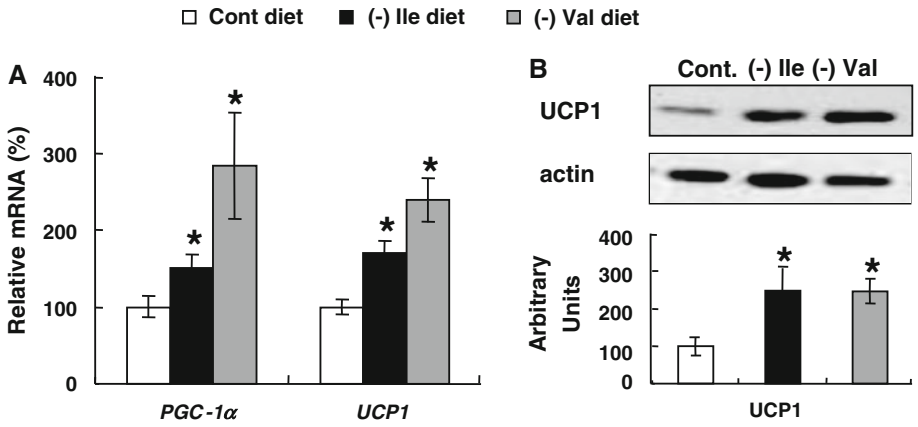


**Fig. 4** UCP1 expression is increased in BAT of isoleucine- and valine-deprived mice. Mice were fed a control, isoleucine-, or valine-deficient diet for 7 days. Data are mean ± SEM of at least two independent real-time PCR experiments (**a**) or western blot (**b**) with mice of each diet for each experiment (control diet [$n = 6$]; (−) Ile diet [$n = 6$]; (−) Val diet [$n = 6$]). Statistical significance was determined by the two-tail student $t$ test for the effect of either (−) Ile or (−) Val diet *versus* control diet (*$p < 0.05$) **a** *Ucp1* and *Pgc1* mRNA, **b** UCP1 protein (*top* western blot, *bottom* UCP1 protein relative to actin)

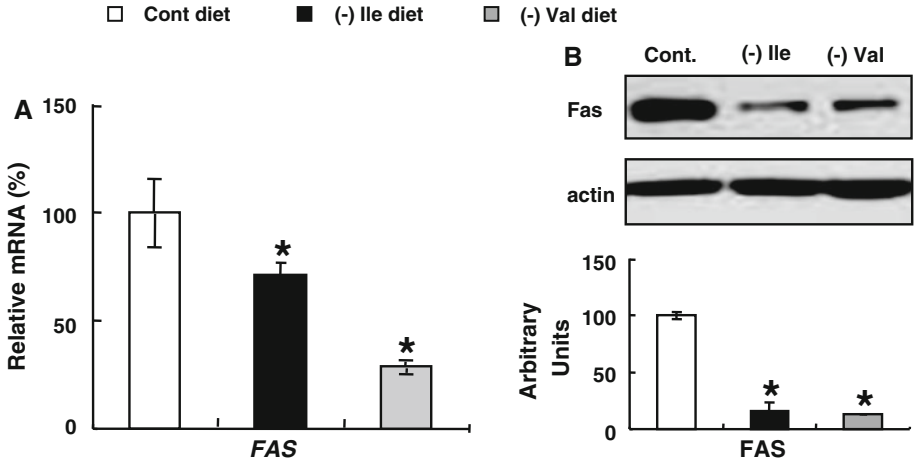


**Fig. 5** FAS expression is suppressed in the livers of isoleucine- and valine-deprived mice. Mice were fed a control-, isoleucine-, or valine-deficient diet for 7 days. Data are mean ± SEM of at least two independent real-time PCR experiments (**a**) or western blot (**b**) with mice of each diet for each experiment (control diet [$n = 6$]; (−) Ile diet [$n = 6$]; (−) Val diet [$n = 6$]). Statistical significance was determined by the two-tail student $t$ test for the effect of either (−) Ile or (−) Val diet *versus* control diet (*$p < 0.05$) **a** *Fas* mRNA, **b** FAS protein (*top* western blot, *bottom* FAS protein relative to actin)

(Cheng et al. 2010) increases body temperature, the effects of isoleucine and leucine on physical activity, however, are different. We previously showed that leucine deprivation had no effect on physical activity, as measured by metabolic cages (Cheng et al. 2010). By contrast, isoleucine deficiency significantly increased physical activity, which may largely contribute to much more increased energy expenditure in these mice. These results suggest that isoleucine may be a more potent regulator for energy homeostasis. It is also possible that isoleucine stimulates certain neuron activities to increase physical activities. It has been shown that physical activity can be regulated by the ventromedial hypothalamus (VMH) (Tou and Wade 2002). For example, studies show that VMH-lesioned rats exhibit hypoactivity (Teitelbaum 1957) and hyperactivity associated with food deprivation is attenuated or absent in VMH-lesioned DIO mice (Greenwood et al. 1974; Challet et al. 1995). Moreover, Amino acid imbalanced diets, including limiting isoleucine content could affect certain neurochemical concentrations in VMH (Gietzen et al. 1989). We therefore speculated that isoleucine deprivation may serve as a signal to affect VMH directly to increase physical activity. This possibility will be investigated in the future study.

As predicted, we found that lipolysis in WAT was substantially stimulated by isoleucine deprivation, which is consistent with leucine deprivation (Cheng et al. 2010). Based on the observation that both isoleucine and leucine

increase expression of genes and proteins related to lipolysis and $\beta$-oxidation, and decreases expression of genes and proteins related to lipogenesis in WAT, we hypothesize that isoleucine deficiency shares similar mechanisms to leucine deprivation in regulating fat loss.

BAT oxidizes fat to produce heat via increasing expression of UCP1, the major isoform expressed in BAT (Feldmann et al. 2009). Ablation of UCP1 results in obese phenotype in mice (Feldmann et al. 2009), whereas upregulation of UCP1 expression results in increased thermogenesis, which helps to protect from fat accumulation and obesity (Li et al. 2000). Thus, UCP1 plays an important role in regulating thermogenesis. Due to increased UCP1 expression, fat is oxidized to produce heat instead of ATP. In BAT of isoleucine-deprived mice, the expression of UCP1 and its upstream gene PGC-1$\alpha$ (Handschin and Spiegelman 2006; Lowell and Spiegelman 2000) are also upregulated, which consistent with the increased body temperature of isoleucine-deprived mice. This suggests that isoleucine deprivation is also able to enhance the function of BAT just like leucine deprivation (Cheng et al. 2010).

Consistent with previous observations in liver (Guo and Cavener 2007), we found that isoleucine deficiency also suppressed lipogenesis in the liver. Our results not only extend a previous in vitro results showing that expression of *Fas* mRNA is suppressed in HepG2 cells incubated in medium deficient of isoleucine (Dudek and Semenkovich 1995), but also suggest the possibility that deficiency of any essential amino acid may inhibit lipogenesis in the liver. Furthermore, we previously showed that decreased lipogenesis in liver is mediated by activation of amino acid sensor general control non-repressed 2 (GCN2) (Guo and Cavener 2007), the mechanism which could also be applied to isoleucine deficiency, as GCN2 is activated by deficiency of any essential amino acid, including isoleucine (Hinnebusch 1994).

The other member of BCAAs is valine. Valine deprivation has almost the same effects on mice as leucine deprivation (Cheng et al. 2010), including body weight, fat mass, food intake, oxygen consumption, and the molecular level changes in WAT, BAT and Liver. The major difference is the significantly decreased body temperature in valine-deprived mice, in contrast to the increased body temperature in either leucine- or isoleucine-deprived mice. Even though we did not see an increase in body temperature, however, we could not exclude the possibility that thermogenesis (as indicated by UCP1 expression) may also contribute to the increased energy expenditure in valine-deprived mice, as the change of body temperature is not always consistent with the change of energy expenditure. For example, even though body temperature is not increased compared with control mice, energy expenditure is increased in high-fat diet-fed mice with ectopic UCP1 expression in the liver (Ishigaki et al. 2005). In addition, mice exhibit increased body temperature while no altered energy expenditure is observed following chronic central amylin treatment (Wielinga et al. 2010). Furthermore, increased UCP1 expression and thermogenesis are not always accompanied by increased body temperature, as those observed in hypoxic/cold acclimated mice (Beaudry and McClelland 2010). Alternatively, it may also be possible that valine deprivation has a direct effect on thermotaxic center. Thus, one of the adaptive changes occurs at this case is to increase UCP1 expression in BAT, thereby to maintain normal body temperature, as many studies reported for the function of UCP1 (Watanabe et al. 2008; Enerback et al. 1997). Both possibilities, however, will be investigated in the future.

It has been shown that insulin and glucagons play important roles in determining the levels of pHSL and p-PKA in WAT (Nishino et al. 2007; Cochet et al. 1999). Insulin levels were much lower in mice maintained on an isoleucine or valine-deficient diet (*data not shown*), suggesting the possible effect of insulin on the increased levels of pHSL and p-PKA in WAT of these mice. The regulation of these changes in WAT by isoleucine or valine deprivation could be very complicated, requiring further investigation, including the possible effect of glucagon.

Autophagy has been shown to be induced by amino acid deprivation (Mordier et al. 2000) and we found increased autophagy-related markers in WAT of mice maintained on an isoleucine or valine-deficient diet. These results suggest that autophagy could at least partially contribute to the decreased fat mass by isoleucine or valine deficiency. The regulatory mechanisms, however, require further investigation.

In summary, isoleucine or valine deprivation has similar effects on decreasing fat mass, which are caused by decreased food intake and increased energy expenditure, thus our current study provide important evidence demonstrating a general effect of BCAAs on body weight and fat mass reduction. BCAAs deficiency may occur during malnutrition in human under protein-restricted diet (Schteingart et al. 1979) or in certain diseases (Malgorzewicz et al. 2008; Qureshi et al. 1998; Canepa et al. 1996). Studies have shown that short-term BCAAs deficiency decreases protein synthesis and stimulates protein breakdown in tissues including liver, WAT and muscle via GCN2 (Anthony et al. 2004; Talvas et al. 2006). Deficiency of essential amino acids last for very long periods, however, may cause serious damage in human. Mechanisms underlying adaptive changes to deficiency of essential amino acids, however, are not fully understood. Our study therefore helps to understand the molecular mechanisms underlying adaptive changes during deficiency of essential

amino acids. Another important issue remains to be investigated is the specificity of our results for BCAAs as opposed to a general deficiency in other non-BCAA essential amino acids. We plan to conduct a systematic analysis to examine the effects of dietary deprivation of other non-BCAA essential amino acids, and furthermore, if these changes are also under control of similar mechanisms.

**Acknowledgments** This work was supported by grants from the Ministry of Science and Technology of China (973 Program 2009CB919001), National Natural Science Foundation (30871208 and 30890043), the Chief Scientist Program of Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (SIBS2008006), the Science and Technology Commission of Shanghai Municipality (08DJ1400601), 2010 Key Program of Clinical Research Center, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (CRC2010005), Key Program of Shanghai Scientific and Technological Innovation Action Plan (10JC1416900). Dr. Feifan Guo was also supported by the One Hundred Talents Program of the Chinese Academy of Sciences.

## References

Anthony TG, McDaniel BJ, Byerley RL, McGrath BC, Cavener DR, McNurlan MA, Wek RC (2004) Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eIF2 kinase GCN2. J Biol Chem 279(35):36553–36561

Beaudry JL, McClelland GB (2010) Thermogenesis in CD-1 mice after combined chronic hypoxia and cold acclimation. Comp Biochem Physiol 157(3):301–309

Canepa A, Perfumo F, Carrea A, Menoni S, Trivelli A, Delucchi P, Gusmano R (1996) Nutritional status in children receiving chronic peritoneal dialysis. Perit Dial Int 16(Suppl 1):S526–S531

Challet E, Le Maho Y, Malan A (1995) Locomotor activity and utilization of energy reserves during fasting after ventromedial hypothalamic lesions. Physiol Behav 58(2):257–264

Cheng Y, Meng Q, Wang C, Li H, Huang Z, Chen S, Xiao F, Guo F (2010) Leucine deprivation decreases fat mass by stimulation of lipolysis in white adipose tissue and upregulation of uncoupling protein 1 (UCP1) in brown adipose tissue. Diabetes 59(1):17–25

Cochet N, Meister R, Florant GL, Barre H (1999) Regional variation of white adipocyte lipolysis during the annual cycle of the alpine marmot. Comp Biochem Physiol 123(3):225–232

Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, Seeley RJ (2006) Hypothalamic mTOR signaling regulates food intake. Science 312(5775):927–930

Doi M, Yamaoka I, Nakayama M, Mochizuki S, Sugahara K, Yoshizawa F (2005) Isoleucine, a blood glucose-lowering amino acid, increases glucose uptake in rat skeletal muscle in the absence of increases in AMP-activated protein kinase activity. J Nutr 135(9):2103–2108

Doi M, Yamaoka I, Nakayama M, Sugahara K, Yoshizawa F (2007) Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis. Am J Physiol 292(6):E1683–E1693

Dudek SM, Semenkovich CF (1995) Essential amino acids regulate fatty acid synthase expression through an uncharged transfer RNA-dependent mechanism. J Biol Chem 270(49):29323–29329

Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP (1997) Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature 387(6628):90–94

Feldmann HM, Golozoubova V, Cannon B, Nedergaard J (2009) UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab 9(2):203–209

Ferre P (2004) The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. Diabetes 53(Suppl 1):S43–S50

Fujioka S, Matsuzawa Y, Tokunaga K, Tarui S (1987) Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity. Metab Clin Exp 36(1):54–59

Gietzen DW, Leung PM, Rogers QR (1989) Dietary amino acid imbalance and neurochemical changes in three hypothalamic areas. Physiol Behav 46(3):503–511

Greenberg AS, Shen WJ, Muliro K, Patel S, Souza SC, Roth RA, Kraemer FB (2001) Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. J Biol Chem 276(48):45456–45461

Greenwood MR, Quartermain D, Johnson PR, Cruce JA, Hirsch J (1974) Food motivated behavior in genetically obese and hypothalamic-hyperphagic rats and mice. Physiol Behav 13(5):687–692

Guo F, Cavener DR (2007) The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. Cell Metab 5(2):103–114

Handschin C, Spiegelman BM (2006) Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. Endocr Rev 27(7):728–735

Hinnebusch AG (1994) The eIF-2 alpha kinases: regulators of protein synthesis in starvation and stress. Semin Cell Biol 5(6):417–426

Ikehara O, Kawasaki N, Maezono K, Komatsu M, Konishi A (2008) Acute and chronic treatment of L-isoleucine ameliorates glucose metabolism in glucose-intolerant and diabetic mice. Biol Pharm Bull 31(3):469–472

Ishigaki Y, Katagiri H, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Shimosegawa T, Sakoda H, Asano T, Oka Y (2005) Dissipating excess energy stored in the liver is a potential treatment strategy for diabetes associated with obesity. Diabetes 54(2):322–332

Jakesz R, Smith CA, Aitken S, Huff K, Schuette W, Shackney S, Lippman M (1984) Influence of cell proliferation and cell cycle phase on expression of estrogen receptor in MCF-7 breast cancer cells. Cancer Res 44(2):619–625

Kimball SR, Jefferson LS (2006) Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. J Nutr 136(1 Suppl):227S–231S

Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 169(3):425–434

Layman DK, Walker DA (2006) Potential importance of leucine in treatment of obesity and the metabolic syndrome. J Nutr 136(1 Suppl):319S–323S

Lee Y, Yu X, Gonzales F, Mangelsdorf DJ, Wang MY, Richardson C, Witters LA, Unger RH (2002) PPAR alpha is necessary for the lipopenic action of hyperleptinemia on white adipose and liver tissue. In: Proceedings of the National Academy of Sciences of the United States of America 99, vol 18, pp 11848–11853

Li B, Nolte LA, Ju JS, Han DH, Coleman T, Holloszy JO, Semenkovich CF (2000) Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. Nat Med 6(10):1115–1120

Loftfield RB, Harris A (1956) Participation of free amino acids in protein synthesis. J Biol Chem 219(1):151–159

Lopez N, Sanchez J, Pico C, Palou A, Serra F (2010) Dietary L-leucine supplementation of lactating rats results in a tendency to increase lean/fat ratio associated to lower orexigenic neuropeptide expression in hypothalamus. Peptides 31(7):1361–1367

Lowell BB, Spiegelman BM (2000) Towards a molecular understanding of adaptive thermogenesis. Nature 404(6778):652–660

Malgorzewicz S, Debska-Slizien A, Rutkowski B, Lysiak-Szydlowska W (2008) Serum concentration of amino acids versus nutritional status in hemodialysis patients. J Ren Nutr 18(2):239–247

Maltese WA, Reitz BA, Volpe JJ (1981) Effects of isoleucine deprivation on synthesis of sterols and fatty acids in LM-cells. J Biol Chem 256(5):2185–2193

Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G, Melchionda N (2001) Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes 50(8):1844–1850

Matthias A, Ohlson KB, Fredriksson JM, Jacobsson A, Nedergaard J, Cannon B (2000) Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty scid-induced thermogenesis. J Biol Chem 275(33):25073–25081

Meister B (2007) Neurotransmitters in key neurons of the hypothalamus that regulate feeding behavior and body weight. Physiol Behav 92(1–2):263–271

Mordier S, Deval C, Bechet D, Tassa A, Ferrara M (2000) Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway. J Biol Chem 275(38):29900–29906

Nairizi A, She P, Vary TC, Lynch CJ (2009) Leucine supplementation of drinking water does not alter susceptibility to diet-induced obesity in mice. J Nutr 139(4):715–719

Nishimura J, Masaki T, Arakawa M, Seike M, Yoshimatsu H (2010) Isoleucine prevents the accumulation of tissue triglycerides and upregulates the expression of PPARalpha and uncoupling protein in diet-induced obese mice. J Nutr 140(3):496–500

Nishino N, Tamori Y, Kasuga M (2007) Insulin efficiently stores triglycerides in adipocytes by inhibiting lipolysis and repressing PGC-1alpha induction. Kobe J Med Sci 53(3):99–106

Noda NN, Ohsumi Y, Inagaki F (2010) Atg8-family interacting motif crucial for selective autophagy. FEBS Lett 584(7):1379–1385

Nogalska A, Sucajtys-Szulc E, Swierczynski J (2005) Leptin decreases lipogenic enzyme gene expression through modification of SREBP-1c gene expression in white adipose tissue of aging rats. Metab Clin Exp 54(8):1041–1047

Qureshi AR, Alvestrand A, Danielsson A, Divino-Filho JC, Gutierrez A, Lindholm B, Bergstrom J (1998) Factors predicting malnutrition in hemodialysis patients: a cross-sectional study. Kidney Int 53(3):773–782

Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z (2007) Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J 26(7):1749–1760

Schoenborn V, Heid IM, Vollmert C, Lingenhel A, Adams TD, Hopkins PN, Illig T, Zimmermann R, Zechner R, Hunt SC, Kronenberg F (2006) The ATGL gene is associated with free fatty acids, triglycerides, and type 2 diabetes. Diabetes 55(5):1270–1275

Schteingart DE, McKenzie AK, Victoria RS, Tsao HS (1979) Suppression of insulin secretion by protein deprivation in obesity. Metab Clin Exp 28(9):943–949

Talvas J, Obled A, Fafournoux P, Mordier S (2006) Regulation of protein synthesis by leucine starvation involves distinct mechanisms in mouse C2C12 myoblasts and myotubes. J Nutr 136(6):1466–1471

Teitelbaum P (1957) Random and food-directed activity in hyperphagic and normal rats. J Comp Physiol Psychol 50(5):486–490

Tou JC, Wade CE (2002) Determinants affecting physical activity levels in animal models. Exp Biol Med 227(8):587–600

Trayhurn P (1979) Thermoregulation in the diabetic-obese (db/db) mouse. The role of non-shivering thermogenesis in energy balance. Pflugers Arch 380(3):227–232

Watanabe M, Yamamoto T, Mori C, Okada N, Yamazaki N, Kajimoto K, Kataoka M, Shinohara Y (2008) Cold-induced changes in gene expression in brown adipose tissue: implications for the activation of thermogenesis. Biol Pharm Bull 31(5):775–784

Watt MJ, Holmes AG, Pinnamaneni SK, Garnham AP, Steinberg GR, Kemp BE, Febbraio MA (2006) Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. Am J Physiol 290(3):E500–E508

Wielinga PY, Lowenstein C, Muff S, Munz M, Woods SC, Lutz TA (2010) Central amylin acts as an adiposity signal to control body weight and energy expenditure. Physiol Behav 101(1):45–52

Wynford-Thomas D, LaMontagne A, Marin G, Prescott DM (1985) Location of the isoleucine arrest point in CHO and 3T3 cells. Exp Cell Res 158(2):525–532

Yousefi S, Perozzo R, Schmid I, Ziemiecki A, Schaffner T, Scapozza L, Brunner T, Simon HU (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat Cell Biol 8(10):1124–1132

Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH (2007) Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. Diabetes 56(6):1647–1654