The Pennsylvania State University
The Graduate School
Department of Cellular & Molecular Physiology

BRANCHED CHAIN AMINO ACIDS SUPPLEMENTATION OF DRINKING WATER
AND DIET INDUCED OBESITY IN MICE

A Thesis in
Physiology
by
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ABSTRACT

Recent studies have implicated amino acids, branched chain amino acids (BCAAs), or Leu alone in improvements of adiposity, body weight control, insulin sensitivity or energy expenditure in humans and rodent models. However others studies have suggested that some of the signaling pathways activated by BCAAs might have negative consequences for obesity and insulin resistance. Therefore it is unclear whether BCAA or Leu supplementation would be beneficial. To begin to address this question we examined the effect of drinking water supplementation of Leu or BCAAs on the development of diet induced obesity (DIO) in C57BL6/J mice. The amino acids were supplemented at close to their saturating concentrations in the drinking water and DIO was induced by feeding a 60% high fat diet. Body weight and food intake, insulin sensitivity, fasting glucose, metabolic rate, body composition and total cholesterol were evaluated. Leu supplementation of drinking water led to a modest but significant rise in fed but not fasted plasma Leu concentrations. However, neither Leu nor BCAA supplementation of drinking water was found to significantly alter body weight increases or food intake during 14 weeks on the high fat diet. Other endpoints such as body composition, insulin sensitivity, cholesterol and energy expenditure were also not affected. Fasting plasma glucose was lower on some days but not others. Taken together the results fail to provide evidence that Leu or BCAAs supplementation of drinking water alone prevents DIO in mice.
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Obesity is a disease resulting from a positive imbalance of energy intake versus energy expenditure. Therapeutic interventions for obesity typically attempt to alter the energy balance equation at the level of energy expenditure or energy intake, for example exercise or dietary restriction respectively [1, 2]. The use of high protein diets or dietary supplementation with branched chain amino acids (BCAAs) or Leu has been explored for therapeutic intervention of obesity and body weight control [3-5]. These interventions have been reported to affect both sides of the energy balance equation, energy intake and expenditure. In the case of energy intake, “Fad” diets with elevated protein have been associated with improved satiety in humans [4]. Similar results have been reported in rodents with either diets containing protein sources rich in BCAAs or with diets supplemented with Leu or BCAAs [3-5]. Similarly, Leu has been shown to decrease food intake through activation of mTOR signaling in the hypothalamus [6] and it might also be expected to affect satiety by stimulating leptin secretion in response to a protein containing meal [7] or by decreasing the orexigenic hormone ghrelin secretion from Gastric cells through mTOR signaling [8].

Supplementation of dietary protein, BCCAs or Leu has also been reported to impact the other side of the energy equation, energy expenditure. The affected component of daily energy expenditure is variously called diet-induced thermogenesis,
the specific dynamic action of food or adaptive thermogenesis [9, 10]. The effects of protein intake on diet-induced thermogenesis do not appear to be solely due to digestion since even the components of digested protein, BCAAs or even Leu alone increase thermogenesis when administered intravenously or orally [11]. This effect may be related to the nutrient signaling effects of amino acids on mTOR, which are largely mediated by Leu [5, 7, 12-19]. Energy consumed by Leu stimulation of protein synthesis may contribute to this increase in energy consumption [4, 5, 11, 12, 20-33].

In light of these findings, it would seem reasonable to expect that Leu supplementation would alleviate obesity. Indeed, Zhang et al. [34] recently reported that Leu supplementation of the water provided to C57BL6/J mice fed a high fat diet increased energy expenditure, resistance to diet induced obesity, insulin sensitivity and plasma total cholesterol. Some, but not all of these changes were consistent with findings we reported in the mitochondrial branched chain amino acid transaminase (BCATm) KO mouse, that has elevated plasma Leu [33]. Similarly, BCAAs or Leu improve body weight control when coupled with exercise or caloric restriction [35, 36]. Shimizu et al. have suggested that BCAA supplementation can decrease the chance of malignancy in obesity related colon cancer animal models and it may be beneficial for colon cancer therapy in obese people by enhancing the insulin sensitivity and decreasing the activity of the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis [37].

These findings support the potential efficacy of BCAA-rich proteins (e.g., whey), BCAAs or Leu for obesity intervention. However in a careful review of the impact of high protein or supplemented dietary interventions, Halton et al. [4] concluded that while
the results of various studies involving dietary supplementation were promising, they were not entirely consistent. Recently Newgard et al. [38] have shown that high fat diet supplemented with BCAA in rats can decrease the rate of weight gain through food intake reduction and it can increase the insulin resistance. They suggested that addition of BCAA acids to high fat diet may have contribution to insulin resistance independent of high fat diet. This suggestion is inferred from their findings in rats solely on high fat diet which were pair fed with rats on both high fat and BCAA. Rats on ad lib high fat diet and BCAA ate less and gained weight less slowly than high fat diet group. Insulin resistance did not emerge in HF pair fed group unlike the rats on ad lib HF diet. They suggested the insulin resistance in HF/BCAA is due to higher chronic activity of mTOR pathway possibly results from stronger activity of JNK pathway upstream to mTOR pathway. However, Zhang et al.[34] have shown no change in food intake of mice fed with high fat diet and supplemented Leucine in their drinking water and have suggested an improvement in insulin resistance due to an increase in the metabolic rate of these mice.

A potential caveat is that persistently over-activating mTOR nutrient signaling through over-nutrition, as might occur with chronic dietary supplementation of amino acids or Leu, has been implicated in Ser phosphorylation of IRS1 leading to insulin resistance and worsening of diabetes [39, 40]. Another paradox is that BCAAs are already elevated in obesity and their concentrations decline in response to a weight loss intervention [41]. Thus further studies are needed to understand the therapeutic potential of BCAAs in obesity.
To begin to address this question we examined the effects of BCAA- and Leu supplementation of drinking water in C57BL6 mice provided a 60% high fat diet. The results of those studies are reported here. Longitudinal changes in body weight, food intake, insulin sensitivity, fasting glucose, metabolic rate, body composition and total cholesterol during the development of diet-induced obesity were measured. We report that neither supplementation affected body weight responses to the high fat diet, in contrast to the findings Zhang et al.[34]. Most of the other parameters measured, with the exception of plasma glucose, were also not affected.
Chapter 2

Experimental Methods

Animal and Treatment Protocol

The PSUCOM Institutional Animal Care and Use Committee approved the animal protocol. Male C57BL/6J mice were purchased from Jackson Laboratories and maintained at our facility for a minimum of one week before the start of treatments. They were maintained on light cycle began at 7 AM and the dark cycle began at 7 PM. Animals were caged separately with food provided *ad libitum* and water or supplemented water provided in graduated cylinders topped with a one hole rubber stopper and metal drinking nipple through the hole. Food and fluid consumption was measured weekly for 2-day periods and averaged. Food remaining in cages and crumbs were weighed and accounted for. A graduated cylinder with a drinking nipple was also always hung on an empty cage to account for spillage (generally 1-2 ml).

Three experiments were conducted with 6-7 week old mice. In each study animals were allocated to one of the following two groups per study: HF (12-15 mice, the control group for each study; HF+ Leu (12-15 mice); HF+BCAA (15 mice). HF mice were provided free access to a high fat diet containing 60% fat calories (D12492, Research Diets Inc, NJ) and *ad libitum* drinking water as per Zhang *et al.* [34] HF+Leu group received the high fat diet plus Leucine containing water. The concentration of Leu was...
150 mM, slightly higher than Zhang et al. [33]; it was begun one week after the high fat diet was initiated. Time 0 is the time the Leu supplementation began. HF+BCAA group received the high fat diet plus BCAAs containing water. Their drinking water was supplemented by 109 mM each of Leu, Ile and Valine. This concentration was lower because the limit of solubility was altered when all three are dissolved. Leu intake was calculated from the fluid and food concentration (2.3 g Leu/100 g chow). BCCAs provided as crystals were ground to a fine powder with a ceramic mortar and pestle to improve solubility.

**Blood Glucose and Insulin Sensitivity**

Blood glucose concentration was measured from tail vein with Glucometer ONETOUCH Ultra (Life Scan, Johnson& Johnson). Insulin Tolerance Tests were performed in 5hr fasted mice. Mice were injected intraperitoneally with 0.75 mU/g body weight of insulin (human insulin, Eli Lilly) and blood glucose was measured at 0, 30, 60, 90 and 120 min after injection.
Energy Expenditure and Activity

Energy expenditure was assessed using indirect calorimetry (Oxymax, Columbus Instruments). Constant airflow (0.6 l/min) was drawn through the chamber and monitored by a mass-sensitive flow meter. The concentrations of oxygen and carbon dioxide were monitored at the inlet and outlet of the sealed chambers to calculate oxygen consumption and RER. Each chamber was measured for 1 min at 15 min intervals. Physical activity was measured using infrared technology (OPT-M3, Columbus Instruments). The counts of three-dimensional beam breaking (X total, X ambulatory, and Z) were calculated using movement detection software provided by Columbus Instruments.

Body Composition

Body composition was determined using an LF90 Minispec TD- NMR Spectrometer (Bruker Optics, TX).
Analytical Procedures

Plasma concentrations of triglyceride and cholesterol were measured using a Vitros Chemistry Analyzer (Ortho-Clinical Diagnosis). Plasma amino acids concentrations were measured using fluorometric HPLC methods.

Statistical Analysis

The effects of Leu and BCAA supplementation on body weight, food intake, Leu intake and insulin sensitivity were assessed using ANOVA repeated measures (Prism, GraphPad Software) with Bonferroni post-hoc analyses when appropriate. Body composition, fasting blood glucose concentration, O2 consumption, RER, locomotor activity, cholesterol, triglyceride and amino acids concentration were assessed using a two-tailed t test (InStat3, 6 GraphPad Software). Values are presented as means ± Standard error; p< 0.05 was considered significantly different. Power analysis was performed using the Statmate computer program 8 (Graphpad software). Graphpad Prism software, was used to test the difference in the slope from zero in some experiments using the F-test performed function.
Effect of Leu and BCAA Supplementation on Body Weight, Food Intake and Fluid Intake During the Development of Diet Induced Obesity

Six to seven week old male C57BL/6J mice were used to examine the effect of Leu and BCAA supplementation during a high fat diet challenge. Body weight was measured weekly during two separate experiments studying Leu supplementation (Fig1, panel A) and in one experiment examining the effects of BCAA supplementation (Fig 1, panel B). No significant differences were observed in the body weights when the Leu supplemented mice were compared to control in either experiment (data not shown). The amount of food consumed also did not differ (Fig 2 Panel A). BCAA supplementation, similarly, lacked effects had on either body weight or food intake (Figs 1B and 2B). Neither Leu nor BCAA supplementation (data not shown) affected daily fluid intake. Fig 2A shows that our protocol for Leu supplementation more than doubled the daily Leu intake (Leu in food and water) whereas less than a doubling occurred with the BCAA supplementation (Fig 2B; notably the concentration of Leu in the BCAA containing water was less). Post hoc power analysis of the two Leu experiments indicated they had 95% power to detect a ~13-15% change in body weight in the last few weeks. When the data from these two experiments was combined, it yielded an n of 27 and higher statistical
power (Fig 1A shows the 18 compiled numbers). However this manipulation did not change the conclusion that Leu lacked an effect on body weight.
Figure 1. **Effect of Leu and BCCA supplementation on body weight during high fat feeding.** Panel A: The compiled results from an experiment on 2 separate cohorts are shown. The regular chow diet of 6-7 week old mice was switched to a 60% kcal high fat diet one week before time zero. At time zero, the mice were randomized into two weight-matched groups. HF mice (n=12 or 15) continued to receive high fat diet and water *ad libitum* in graduated cylinders. HF+ Leu mice (n=12 or 15) also receive Leu-supplemented water. Body weight was measured weekly. Panel B: In another study, the experimental group (HF+ BCAA) received water containing all three branched chain amino acids, Leu, Ile and Valine as described in the methods. Symbols indicate 11 mean plus or minus the standard error where bar exceeds the size of the symbol.
Figure 2. **Food and Leu intake during development of diet induced obesity.** Panel A:

The amount of food and fluid consumed was measured for 2 days during Leu supplementation and shown as an average daily value. Leu intake was calculated from the fluid and food (2.3 g Leu/100 g chow) intake.
Figure 2. **Food and Leu intake during development of diet induced obesity.** Panel B:

The amount of food and fluid consumed was measured for 2 days during BCAA supplementation. Symbols indicate mean plus or minus the standard error where bar exceeds the size of the symbol.
Effect of Leu and BCCA Supplementation on Total Cholesterol and Triglyceride Plasma

Concentrations of total cholesterol were measured at week 13 and/or at week 15 after either 5 hrs or overnight food deprivation (Table 1). Leu or BCAA supplementation did not significantly improve the plasma cholesterol concentration however there was a trend toward lower mean values. No significant difference was observed in the plasma triglyceride concentration (77.5±5 mg/dl in HF mice after 5hrs fasting vs. 73±3.1 mg/dl in HF+Leu. 59.5±2.8 mg/dl in HF mice after overnight fasting vs. 62±4.3 mg/dl in HF+Leu).

Effect of Leu and BCCA Supplementation on Fasting Glucose and Insulin Sensitivity

Fasting plasma glucose concentrations were determined from various random samplings or from time 0 blood draws for ITT experiments. Consistent with several previous reports of effects on plasma Leu on glucose, the plasma glucose concentration was 9-12 percent lower on several occasions, or trended to be lower, in the supplemented groups; but this was not always the case (Table 2). An insulin tolerance test was performed after 14 weeks of high fat feeding and Leu supplementation (Figure 3A) or BCAA supplementation (Figure 3B) to assess insulin sensitivity. No significant changes in insulin sensitivity were suggested by these tests.
Table 1. **Effect of Leu and BCAA supplementation on Total Cholesterol**

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
<th>Total Cholesterol, mg/dl</th>
<th>n</th>
<th>p-value</th>
<th>Nutritional state</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF+Leu</td>
<td>HF</td>
<td>153.5±8</td>
<td>10</td>
<td>0.62</td>
<td>‡Overnight fasted</td>
</tr>
<tr>
<td></td>
<td>HF+Leu</td>
<td>146.6±6.3</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HF</td>
<td>191.1±5.5</td>
<td>15</td>
<td>0.15</td>
<td>†5 hrs fasted</td>
</tr>
<tr>
<td></td>
<td>HF+Leu</td>
<td>178.9±6.1</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF+BCAA</td>
<td>HF</td>
<td>153.9±4.6</td>
<td>11</td>
<td>0.26</td>
<td>‡Overnight fasted</td>
</tr>
<tr>
<td></td>
<td>HF+BCAA</td>
<td>144.8±6.3</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Results are mean and standard error, n is the number of determinations which was lower than the n of mice because of insufficient plasma samples in some cases or because some animals were allocated to a different fasting regimen.

†Mice were food deprived for 5 hours.

‡Mice were food deprived overnight, mice in HF+Leu continued to receive leucine in the water and in HF+BCAA continued to receive BCAA in the water.
Glucose measurements were made on plasma samples from multiple blood draws collected at different times, including time 0 blood from ITT, during weeks 11-14 of the experiments. The results are mean and standard errors.

† Mice in HF+Leu group continued to receive leucine in the water.

‡ Mice were food deprived for 5 hours, mice in HF+BCAA group were switched to water.

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
<th>Glucose, mg/dl</th>
<th>n</th>
<th>p-value</th>
<th>Nutritional State</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF+Leu</td>
<td>HF</td>
<td>249±4.6</td>
<td>12</td>
<td>0.049</td>
<td>† 5 hrs fasted</td>
</tr>
<tr>
<td></td>
<td>HF+Leu</td>
<td>228±9.0</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF+Leu</td>
<td>HF</td>
<td>281±9</td>
<td>12</td>
<td>0.026</td>
<td>† 5 hrs fasted</td>
</tr>
<tr>
<td></td>
<td>HF+Leu</td>
<td>246±12</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF+Leu</td>
<td>HF</td>
<td>198±7.5</td>
<td>12</td>
<td>0.03</td>
<td>† overnight fasted</td>
</tr>
<tr>
<td></td>
<td>HF+Leu</td>
<td>173±7.8</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF+Leu</td>
<td>HF</td>
<td>217±10.8</td>
<td>15</td>
<td>0.105</td>
<td>5 hrs fasted</td>
</tr>
<tr>
<td></td>
<td>HF+Leu</td>
<td>194±8.5</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF+Leu</td>
<td>HF</td>
<td>211 ± 8.9</td>
<td>15</td>
<td>0.2</td>
<td>5 hrs fasted</td>
</tr>
<tr>
<td></td>
<td>HF+Leu</td>
<td>228 ± 9.7</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF+BCAA</td>
<td>HF</td>
<td>219.6±7.1</td>
<td>12</td>
<td>0.27</td>
<td>‡ 5 hrs fasted</td>
</tr>
<tr>
<td></td>
<td>HF+BCAA</td>
<td>231.2±7.9</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Effect of Leu or BCAA supplementation on insulin sensitivity. An Insulin Tolerance Test was performed after 14 weeks of high fat diet in plus or minus either Leu (Panel A) or BCAA (panel B) groups. Insulin (0.75 mU per gram body weight) was injected intraperitoneally after 5hrs of fasting. Symbols indicate mean and standard error.
Effect of Leu and BCAA Supplementation on VO₂, RER and Locomotor Activity

O₂ consumption, RQ (RER) and activity were measured after 14 weeks of Leu supplementation (Figure 4A) or 16 weeks of BCAA supplementation (Figure 4B). Neither supplementation changed the O₂ consumption, RER and activity. Data was analyzed for differences over the whole day or during just the light or dark cycle. These analyses did not change the conclusion that there were no significant differences in these parameters. Light to dark cycle changes in activity, RQ or VO₂ were not affected by the supplementations.

Effect of Leu Supplementation on Plasma Amino Acids Concentration

Fig 5 shows the plasma concentration of amino acids in the fed state. The fed plasma Leu concentration was 76% higher in HF+Leu mice compared to control, whereas the [Valine], [Glycine] and [Arginine] were significantly lower in HF+Leu mice than in HF mice. The increase in plasma Leu is comparable to that observed by Zhang et al.[34].

Effect of Leu on Body Composition

Body composition by TD- NMR was determined after 8 and 16 weeks of Leu supplementation (Table 3). Chronic Leu supplementation did not change the proportion
of fat or lean tissue in the HF+Leu compared to the HF group. The total body weight was again not significantly different between two groups when these measurements were made.
Figure 4. **Effect of Leu and BCAA supplementation on oxygen consumption, RER and locomotor activity.** Panel A. Oxygen consumption (VO2, top left panel), respiratory exchange ratio (RER, top right panel) and locomotor activity (bottom panel) were measured after 14 weeks of high fat feeding in different groups of mice described in Fig 1 legend. Symbols indicate mean and standard error.
Figure 4. Effect of Leu and BCAA supplementation on oxygen consumption, RER and locomotor activity. Panel B. Oxygen consumption (VO2, top left panel), respiratory exchange ratio (RER, top right panel) and locomotor activity (bottom panel) were measured after 16 weeks of high fat feeding in different groups of mice described in Fig 1 legend. Symbols indicate mean and standard error.
Figure 5. Effect of Leu supplementation on plasma concentrations of amino acids.

After 14 weeks of high fat feeding, blood samples were collected from HF and HF+Leu mice during feeding. Plasma concentrations of amino acids were measured by HPLC.

Symbols indicate mean and standard error, * indicates p < 0.05
Table 3. Effect of Leu on H1-NMR Body composition

<table>
<thead>
<tr>
<th>Week of study</th>
<th>Week 8</th>
<th>Week 16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Condition</strong></td>
<td><strong>HF</strong></td>
<td><strong>HF+Leu</strong></td>
</tr>
<tr>
<td>Lean, g</td>
<td>24.2± 0.5</td>
<td>24.4± 0.5</td>
</tr>
<tr>
<td>Fat, g</td>
<td>10.1± 0.6</td>
<td>9.2± 0.8</td>
</tr>
<tr>
<td>Fluid, g</td>
<td>3.6± 0.1</td>
<td>3.5± 0.1</td>
</tr>
</tbody>
</table>

* Results are mean and standard error
Variability of Body Weight and Correlation of Body Weight and Plasma Total Cholesterol

Fig 6 (top panel) shows individual body weights after 15 wks of Leu supplementation and high fat feeding. Standard deviations for the end body weights ranged from ~4-6.2 gm within each group of the various experiments. The variability was of sufficient magnitude that significant differences were observed between the body weights of the five lightest and heaviest animals [c.f., n=5 from Zhang et al. [34]] irrespective of treatment. Notably, there was no correlation between the starting body weights (which were purchased with a narrow range) and body weights after high fat challenge in either group. Zhang et al.[34] reported that Leu supplementation had a body weight independent effect of plasma total cholesterol, however the n was only five. Fig 6 (bottom panel) shows that plasma total cholesterol correlated with body weight, again, irrespective of treatment group. In contrast to Zhang et al.[34], we observed a high degree of correlation either when the HF and HF+Leu data was analyzed separately or together (Fig 6). Thus plasma cholesterol was not body weight independent.
Figure 6. **Individual body weights after high fat diet challenge and relationship of body weight to plasma total cholesterol.** Panel A: Individual body weights are plotted for the HF and HF+Leu group after 15 weeks of Leu supplementation. As indicated these data are from the second Leu supplementation experiment (compare Fig 1A). The symbols represent individual body weights and the line is the mean body weight. “High 5” and “High 5(+L)” are plots of the highest five individual body weights (n=5) for the HF and the HF+Leu group, respectively. “Low 5” and “Low 5(+L)” are plots of the lowest five individual body weights (n=5) for the HF and the HF+Leu group, respectively. ** indicates a significant difference (P<0.01) between the High and Low values within or across each group. Panel B: Correlation between individual week 15 body weights and plasma total cholesterol concentrations. The p value is from an F test used to test the hypothesis that the slopes were different from zero.
Chapter 4

Discussion

We examined the effect of supplementing drinking water with saturating concentrations of Leu (~150 mM) or BCAAs on diet induced obesity in C57BL6 mice. Neither Leu nor BCAA supplementation affected body weight or body composition in mice fed a high fat diet. Not surprisingly other obesity-related metabolic endpoints were similarly unaffected by these interventions. Leu or BCAAs provided by approach was insufficient to exert previously reported effects of Leu/BCAAs on food intake or body weight control. We attribute this to a several factors including the method and amount of the supplementation as well as statistical power. In addition in some previous studies Leu supplementation was coupled to some other intervention such as dietary restriction [35, 36]. Here the mice were fed ad libitum.

Effect of supplementation on body weight production on the high fat diet. Part of the reason for the lack of effects of Leu/BCAA supplementation on body weight in our study may related to the modest elevations in plasma leucine induced by this method of administration. Because the solubility of the BCAAs in water is relatively limited compared to other amino acids, the plasma concentrations of Leu achieved in our supplementation, though statistically significant, was relatively modest (less than 2-fold in random fed mice). In comparison, in meal trained rats, Leu concentrations increase ~3-4 fold during the meal. In BCATm KO mice, which had strong effects on insulin sensitivity and body weight, Leu concentrations are typically elevated ~5-32 fold
depending on the diet [33]. Therefore the concentration of leucine may not be great enough to observe changes in body mass seen with other studies. [7, 42-45]. Newgard et al.[38] have shown weight loss in rats fed with pellets of high fat diet and BCAA due to a decrease in food intake. However we did not see any change in food intake of mice fed with high fat diet and supplemented BCAA in their drinking water. That difference may be due to using different species and method of BCAA supplementation. In our experiment BCAA and Leucien were dissolved in drinking water in order to decrease the possibility of taste aversion arising from food pellets. Another reason for the lack of effect may be related to statistical power and the variable response of C57BL6 mice to diet induced obesity. For example, whereas we did not observe a change in body weight after 8-15 weeks of Leu or BCAA supplementation in mice provided a 60% fat diet, Zhang et al. [34] reported an approximate 8-10% reduction in body weight in response to Leu supplementation. This difference is not due to the diet we used, mouse strain, source of mice or Leu concentrations, which were the same or similar. Since the high fat diet produced similar body weight production in the control mice in both studies, that too does not seem to be an issue. The fed amino acid plasma concentrations we observed also appeared similar to Zhang et al. [34]. However, one difference was in the n used for each of the studies, we used 12-15 mice per group whereas Zhang et al.[34] used 5. An n=5 seems rather small to us because several groups have reported unexplained inter-mouse variability in the responses of C57BL6 mice to high fat diet feeding [46-49]. For example, Koza et al. [47] reported end body weights between ~28-52 gm (discerned from their graphics) in a study involving the response of 107 male C57BL6 mice to high fat feeding. This variability in
the response to DIO appeared to arise from epigenetic factors, rather than experimental conditions. Such variability may be more apparent when larger numbers of mice are used. Based on their reported n=5 and standard deviations, the Zhang et al.[34] study only had ~50% power to reproducibly observe a 3.8 g difference in body weight. This would generally be considered to be underpowered. Post hoc analysis of our data indicates it was sufficiently powered (95% power) to detect a ~13-15% change in body weight. While the chance to observe a smaller change in body weight was increased by repeating the study, we cannot rule out the possibility that Leu or BCAA supplementation actually does cause an ~8-10% lowering (~3.8 g) in body weight as suggested by Zhang et al. [34]. Such a lowering would be clinically relevant, however we predict that a well powered study that could reproducibly show such a change would require around 50 mice per group based on the standard deviations we observed in three separate studies. [7, 42-45]. Newgard et al.[38] have shown a decrease in food intake in rats fed with pellets of high fat diet and BCAA, however we did not see any change in food intake of mice fed with high fat diet and supplemented BCAA in their drinking water. That difference may be due to using different species and method of BCAA supplementation. In our experiment BCAA and Leucien were dissolved in drinking water in order to decrease the possibility of taste aversion arising from food pellets

*Oxygen consumption and plasma cholesterol.* In the present study rates of oxygen consumption typically within the range of 2000-3000 ml/kg/hr were observed consistent with our previous study [33] and typical for mice of this strain and diet [e.g., 47, 48]. These were not affected by Leu supplementation. On the other hand Zhang et al. [33],
reported that Leu supplementation increased VO$_2$ by around 15%. Their values for VO$_2$ were in the range of ~7000-11,000 ml/kg/hr with light to dark cycle shifts in the range of 2000-2500 ml/kg/hr. These seem too high in our experience, suggesting some systematic problem with the calorimetry in their study. Neither BCAA nor Leu supplementation lowered plasma cholesterol significantly in our experiments, in contrast to Zhang et al. [33]. In addition, we did observed a high degree of correlation between plasma cholesterol and body weight production on the high fat diet. Thus, going back to type 1 error, if we had chosen five HF diet responders and five HF resistant mice at the beginning of the study randomly, a difference in the plasma cholesterol would have been expected and the values might be sufficiently clustered as to not reveal a significant correlation between end body weights and plasma cholesterol concentrations after high fat feeding.

**Summary.** In conclusion, our study suggests that neither BCAA nor Leu supplementation of water leads to a significant reduction in body weight or plasma cholesterol. Because of the response variability of C57BL6 mice to diet induced obesity, far more mice would be needed to reproducibly see a clinically significant 8-10% change in body weight after high fat diet supplementation. Such effects might be easier to see with another study design (e.g., a longitudinal design in DIO responders) or when some other intervention is added such as diet restriction or exercise [34, 35]. Alternatively, higher plasma Leu concentrations may be needed throughout the day to see a significant effect on body weight, since plasma Leu elevation achieved by supplementation of the drinking water is not as high as that observed in the BCATm KO mice which are incapable of peripheral BCAA metabolism. To achieve these levels an inhibitor of
BCATm might be needed in addition to supplementation. We are hopeful that conditions can be found to demonstrate BCAAs have a beneficial effect on body weight and adiposity, but at present lack evidence for that based on the present study.
REFERENCES


