Alloisoleucine Differentiates the Branched-chain Aminoacidemia of Zucker and Dietary Obese Rats
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Objective: Circulating branched-chain amino acids (BCAAs) are elevated in obesity and this has been linked to obesity comorbidities. However, it is unclear how obesity affects alloisoleucine, a BCAA and pathognomonic marker of branched-chain keto acid dehydrogenase complex (BCKDC) disorders. It has been previously established that obese Zucker rats exhibit BCKDC impairments in fat and other tissues, whereas BCKDC impairments in adipose tissue of DIO rats are compensated by increased hepatic BCKDC activity. Therefore, alloisoleucine was investigated in these two obesity models.

Methods: Amino acids were extracted from plasma and measured using ultra performance liquid chromatography mass spectrometry.

Results: Plasma alloisoleucine was 238% higher in obese compared to lean Zucker rats. This elevation was greater than that of other BCAAs (107-124%). DIO rats had no significant change in alloisoleucine, despite elevations in other BCAAs (15-66%).

Conclusions: Alloisoleucine was elevated in obese Zucker but not DIO rats consistent with known global impairments of BCKDC in Zucker but not DIO rats. Cytotoxic branched-chain ketoacids (BCKAs) accumulate in genetic disorders affecting BCKDC. BCKAs increase reactive oxygen species, stress kinase activation, and mitochondrial dysfunction. Inasmuch as these factors underlie obesity comorbidities, it may be important to identify obese individuals with elevated alloisoleucine.

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Introduction
Branched-chain amino acid (BCAA) supplementation is frequently linked to endpoints associated with good health including satiety, diet-induced thermogenesis, improved glycemia, and lean body mass (1, 2). Paradoxically, BCAAs are elevated in obesity where they have been described as a “metabolic signature” for insulin resistance, associated with glucose intolerance, and future diabetes (3, 4).

In different types of obesity, distinct processes affecting BCAA rates of appearance or disappearance (food intake, protein turnover, oxidation, protein synthesis, and excretion) may be altered and their contribution or relative direction of change may differ between models (5-8) and perhaps individuals. On the other hand, a consistent observation across obesity models and humans is a loss of BCAA metabolism in adipose tissue (5). It has been hypothesized therefore that loss of BCAA metabolism in fat may contribute to the branched-chain aminoacidemia of obesity (5, 7-9).

While fat appears to be a quantitatively important site of BCAA metabolism, it may be questioned whether a defect in BCAA metabolism in a single tissue is sufficient to elevate whole body BCAA concentrations. That is because (a) BCAAs are rapidly converted to branched-chain keto acids (BCKAs) in many peripheral tissues and can circulate in the blood to be metabolized elsewhere, (b) the branched-chain keto acid dehydrogenase complex (BCKDC) activity responsible for that metabolism is distributed across many tissues and (c) is substrate activated. Thus a systematic loss of BCAA metabolism in maple syrup urine disease (MSUD) or models thereof can be largely alleviated by transplantation of the liver or adipose tissue (reviewed in Ref. 10). Thus a fat specific impairment of BCKDC in obesity could theoretically be compensated for by increased activity in other tissues. This scenario is exemplified in DIO rats where BCKDC is impaired in fat, but in liver the activity is increased more than two-fold (6, 7). In contradistinction, in other models (e.g., ob/ob mice, ZDF rats, and Otsuka Long Evans Tokushima Fatty rats), hepatic BCKDC is impaired by obesity (9, 11).
11). In obese Zucker rats, impairments of BCKDC in fat were matched by ~50% activity losses in liver and all other peripheral tissues tested (8, 9).

Whether BCKDC dysfunction extends to multiple tissues in obesity is an important question because systematic impairment of BCKDC could contribute to obesity comorbidities. Mutations in several BCKDC subunits (including BCKDHA) or BCKDC phosphatase (PPm1K, a.k.a. PP2Cm) can cause various forms of MSUD. Even in untreated milder forms of MSUD, potentially cytotoxic BCKAs circulate and accumulate in tissues. BCKA addition to MSUD cells activates stress kinases (JNK and p38), increases ROS generation, and causes mitochondrial dysfunction (12, 13). These factors are linked to insulin resistance, diabetes, and cardiovascular disease (12, 13). Consistently, both BCKDHA (encoding the subunit regulated by phosphorylation) and PPm1K (the BCKDHA phosphatase) are primary obesity/diabetes susceptibility genes (14-16), and PPm1K has also been implicated in cardiovascular disease (17). Thus, subjects with type II diabetes had lower beta cell PPm1K, and partial silencing of PPm1K in beta cells impaired glucose stimulated insulin secretion (14). Furthermore, a specific allelic variation near PPm1K was associated with elevated BCAAs along with poorer glycemic and weight loss responses in the POUNDS LOST trial (16). Thus global BCKDC impairment in obesity could potentially contribute, along with other factors, to the development of obesity co-morbidities that higher concentrations of BCAAs appear to portend.

A practical means to identify obese types with partial global BCKDC impairments as opposed to those restricted to adipose tissue could be useful. Here we explored using alloisoleucine, the pathognomonic marker of MSUD, for that purpose. Due to its long half-life, alloisoleucine is not significantly impacted by acute factors such as nutritional status (18). Plasma alloisoleucine below the cut-off used for MSUD diagnosis, typically 2μM (19), are not usually measured with standards or reported by clinical laboratories, so it is unknown how alloisoleucine might vary due to obesity within the “normal range”. Given that impairments in BCKDC were observed in multiple tissues of obese Zucker rats, but were restricted to fat and compensated by increased hepatic activity in obese DIO rats (6, 8, 9), we tested the hypothesis that alloisoleucine might be elevated in Zucker but not DIO rats.

Methods

Animals

All procedures were approved by the Penn State Hershey Institutional Animal Care and Use Committee (IACUC). Excess, banked (~80°C) heparinized plasma from two previous rat studies were used here. In both studies the plasma was collected about 3-4 h after the end of the dark cycle. In the Zucker rat study, male obese (fa/fa, 455 ± 5 g body weight, n = 10) and lean control (Fa/?, 280 ± 3 g, n = 10) rats from Charles River Laboratories (Cambridge, MA) were provided Teklad 2018 diet, a low fat diet, and maintained as previously described (8). The DIO samples were from ad libitum-fed Sprague-Dawley rats (Charles River Laboratories) maintained for more than 20 weeks on the same lean chow (396 ± 12 g body weight, n = 10) as the Zucker rats (Teklad 2018) or a 60% fat diet (Research Diets D12492) leading to DIO (867 ± 13 g final body weight, n = 10).

Ultra pressure liquid chromatography mass spectrometry

Amino acids and an internal standard were extracted from plasma using a Waters Oasis MCX 1 cc solid phase vacuum extraction system according to the manufacturer’s instructions. Separation and analysis of alloisoleucine, Ile, Leu, and Val was then performed as previously described (10) on a Waters Synapt HDMS hybrid QTOF with Ion Mobility, housed in the Penn State College of Medicine Macromolecular Core Facility. Two injection volumes were used for each sample to maintain mass spectrometry (MS) signals within linear range, 10 μl for alloisoleucine, and 0.25 μl for the other amino acids. The standard curve included amino acid concentrations of 0.1 μM and above.
Statistical analysis
Data are expressed as mean ± SEM. Two-tailed unpaired t-tests and data correlation analyses was performed using Graphpad Prism 6.0 software (La Jolla, CA); P < 0.05 was considered significant.

Results
BCAAs, Phe, and alloisoleucine were measured in plasma by ultra pressure liquid chromatography mass spectrometry. Compared to lean rats, obesity elevated BCAAs by 107-124% on average in plasma from obese Zucker rats (lean vs. obese: Ile, 42 ± 3 vs. 87 ± 3 μM; Leu, 85 ± 5 vs. 190 ± 10 μM; Val, 125 ± 7 vs. 266 ± 9 μM; n = 9/group, P < 0.001). Phenylalanine was elevated 24% (67 ± 1 vs. 83 ± 2 μM, P < 0.001). Alloisoleucine is the R-epimer of Ile, formed as a rare side reaction during the reversible transamination of Ile or S-ketomethylvalerate (S-KMV) (20). The mechanism of its formation is schematically shown in Figure 1. In obese Zucker rats, alloisoleucine was elevated 238% (Figure 2A), with some individual values between 1.3 and 1.8 μM (not shown). No significant correlation was observed between plasma Ile and alloisoleucine concentrations in either lean or obese rats (data not shown).
Amino acids were also measured in DIO rats. BCAAs in the DIO rats were elevated 15-66% as follows for lean vs. obese: Ile, 51 ± 3 vs. 77 ± 3 μM (P < 0.001); Leu, 136 ± 7 vs. 157 ± 4 μM (P < 0.05); Val, 124 ± 6 vs. 206 ± 9 μM (P < 0.001). Phenylalanine was unaltered (75 ± 3 vs. 73 ± 2 μM, N.S.). Alloisoleucine was not changed by obesity in DIO rats (Figure 2B), in contrast to Zuckers (Figure 2A). A schematic model showing why alloisoleucine may be elevated in obese Zucker but not DIO rats is shown in Figure 2C.

Discussion

In this article, we have shown that the BCAA, alloisoleucine, is elevated in obese Zucker rats but not DIO rats. While all of the measured alloisoleucine concentrations in plasma from obese Zucker rats fell below the 2 μM cut off used for MSUD diagnosis (19), some individual values approached that value. Consistent with dogma that elevations in Ile (pool size) without a systematic loss of metabolism do not elevate alloisoleucine (18), DIO rats had elevated Ile but no significant changes in plasma alloisoleucine.

Most models of obesity, including Zucker and DIO rats, exhibit changes in adipose tissue BCAA metabolism (5, 7). In obese Zucker rats, BCKDC impairments extend to other peripheral tissues (7-9), whereas in DIO rats, while BCKDC is impaired in fat, hepatic BCKDC activity is greatly increased (6). The mechanisms underlying these differences are unknown. It is tempting to speculate that alloisoleucine might be able to differentiate these situations in other models and obese individuals. It seems likely that there may be some obese individuals with global versus tissue selective effects on BCKDC because BCKDHA and PPm1K have been identified as type-2 diabetes and/or obesity susceptibility genes (14-16).

Proportionally, the elevation of alloisoleucine in obese Zucker rats was more than that observed for other BCAAs. Thus the signal to noise ratio of alloisoleucine elevations was greater than other BCAAs in Zucker rat obesity and the elevation appeared to be model selective since it did not change in obese DIO rats. Thus measuring alloisoleucine changes may provide a robust and selective marker of partial global impairments in BCKDC associated with certain types of obesity.

A potential limitation of our hypothesis is that the obese Zucker rats we studied tend to avoid diabetes. However, on another genetic background, ZDF, the same loss of leptin signaling leads to diabetes. Obese diabetic ZDF rats also have been reported to exhibit BCKDC impairments affecting liver (11). A similar limitation is that in a small cohort of diabetes patients, alloisoleucine concentrations on average were not different from non-diabetic subjects; however the upper end of values for diabetic subjects were nevertheless very close to the diagnostic cut-off for MSUD used at the time for that older methodology (18).

Further studies are needed to understand the potential pathological significance of global BCKDC impairments versus those restricted to fat and compensated by liver activity changes in different models of obesity. It also remains to be determined whether alloisoleucine is more robust or has a different pattern or selectivity in longitudinal studies compared to the major BCAAs that correlate with or predict obesity co-morbidities.

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